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# What case for basic research?

*Researchers threatened by the budgetary process in the United States are preaching the virtues of basic research. But even Dr Frank Press's argument will not wash. What should be done?*

The budgetary process in the United States, always confused, is this year causing more than the usual havoc. There is now a serious danger that, whatever is decided, needless damage will be done to important scientific institutions. For although it is customary that the Administration's authority to conduct government business at the beginning of the financial year stems from a continuing and temporary resolution by Congress, the sequence of events this year will provide the new President's men with a licence to spread alarm and despondency among the agencies they control. Some, to their shame, seem to have leaped at the opportunity. But even when the heads of government agencies have been resolute and far-sighted, research managers have been forced to think what has hitherto been unthinkable. The general mood of alarm has spread widely through that part of the American research establishment supported directly by the Administration. Further afield, those who will be looking for research grants from the grant-making agencies are despondent — or are making eyes at the Department of Defense, one of the few agencies of the United States government certain to have more to spend. Whatever damage is done by whatever budget is eventually agreed, the mere process of getting something settled is now certain to leave a scar on the research enterprise. Sooner rather than later, something must be done to put things right.

## Budget

Since the beginning of the year, Congress has been faced with no fewer than three versions of the budget for the year that started on 1 October, beginning with President Carter's valedictory budget which must in retrospect be read as his declaration of how well-heeled everybody would have been if only they had voted for him in sufficient numbers. The framework of the new budget appeared only in March, together with President Reagan's proposals for reductions in the rates of income and corporation taxes that, in what is almost certain to prove a pyrrhic victory, the President carried through Congress early in the summer. Left alone, Congress might well have lived up to form and enacted some kind of budget authority in the usual wild legislative scramble between Thanksgiving Day and Christmas. The trouble, which became apparent during August, is that the March budget coupled with the tax cuts would require the United States to run a deficit of between \$60,000 million and \$100,000 million in the twelve months to next October. Seeking to retain some semblance of Republican virtue in the management of its affairs, the Administration accordingly proposed in September a 12 per cent cut in the discretionary expenditure of all its agencies except the Department of Defense (whose increased budget was cut by \$3,000 million in August) and the National Aeronautics and Space Administration (whose spending is reduced by only 6 per cent this year).

Congress is now trapped in a familiar dilemma. The Administration is right to argue that deficit financing will only sustain inflation and prolong the period when interest rates remain high, but agreeing to the cuts proposed will be unpopular with the voters (who will be going to the polls only a year from now). So members of Congress are cheerfully denying the President the 12 per cent cut he asks for, preferring that he rather than they should incur the odium of appearing mean, which is what will happen if he should veto the inflated appropriations bills. The only quick way out of the impasse that has been created

is that the Administration should agree to a postponement of its plans for the MX missile and the B1 bomber, against which both houses of Congress have raised cogent objections, in which case the inflationary implications of the present budget will seem less threatening. That, however, would be a climb-down for the Administration which is unlikely to be undertaken lightly or quickly. In the meantime, the agony in the research community will continue.

Rumours abound of what the consequences will be. Will it be necessary to close whole laboratories? The Brookhaven National Laboratory is potentially a casualty if its new accelerator is abandoned (see *Nature* 5 November, p.3). And if it turns out that the National Aeronautics and Space Administration has no funds left over from the ill-starred space shuttle, can it make sense to keep the Jet Propulsion Laboratory alive? Thus, almost casually, the fate of powerful components of the American research enterprise are loudly discussed. No wonder that those concerned are making discreet enquiries of the Pentagon about the military tasks they might take on. Not all establishments are as fortunate, however. The laboratories of the Bureau of Drugs whose members have been told their jobs are not of the highest priority (see page 101) will be kept on tenterhooks for many months to come. Elsewhere, even in laboratories whose usefulness is widely acclaimed, research directors are compelled by sheer prudence to draw up lists of people whose jobs may have to go on the insubstantial grounds that, with more than a tenth of the financial year already gone, and while the possibility remains that they may have to reduce expenditure by an eighth before the financial year is over, preparing to fire people soon is more important than the prosecution of an imaginative research programme. This is no way in which to deal with intellectual enterprises. So much, both Congress and the Administration must be given clearly to understand.

## Message

But how? What kind of message is likely to carry weight with an Administration apparently less sensitive than its predecessors to intellectual interests and a Congress preoccupied, as always, with the problems of its own reelection? Dr Frank Press's colloquium at the National Academy of Sciences two weeks ago (see *Nature* 5 November, p.4) was a first attempt at putting the case for basic research to the Administration, but is unlikely to carry much weight. For the plain truth is that in the United States, as in Britain for the past several years, basic research as such has come to be regarded as effete, a kind of luxury. Everybody knows that the pursuit of a deeper understanding of natural phenomena gives great pleasure to those directly engaged in it, while even the most cynical legislators are prepared to hail the award of a Nobel prize to a fellow-countryman as a mark of their own percipience (which is naturally magnified a hundredfold if the prizewinner should be a constituent). To do them justice, however, legislators are also properly preoccupied with the larger question of how they can be held by their constituents or anybody else to have behaved responsibly if they condone inflation and thus, by extension, the erosion of the value of property, the undermining of established institutions and the setting of a trend towards the fly-by-night. Dr Press's "consensus statement" made a seemingly acknowledgement of the United States Administration's economic problems. It went disastrously awry in asking that if government support for



science and technology had to be reduced, basic research should be given priority over development and demonstration. Even the Majority Leader in the Senate, Senator Howard Baker, has helped in the past few days to breathe new life into the most spectacular demonstration of all — the technically antiquated fast reactor at Clinch River. It is, however, unlikely that Senator Baker did not know of Dr Press's colloquium. More probably, he did not care.

## Incorrigibility

So are the Senator Bakers of this world incorrigible? Or is it that those engaged creatively in basic research are indefinitely and repeatedly dispensable? These questions are not easily dealt with. Most politicians will accurately calculate that if the population of those now engaged on basic research is so eager to continue with that pursuit, the recruitment of a substitute should be relatively easy once it again becomes affordable. Moreover, the calculation is entirely correct. There will never be a shortage of people willing to deepen understanding if offered the chance. What is in question is their capacity to do so in a vacuum. The experience since the Second World War of a variety of nation-states self-conscious about their conduct of basic science has shown convincingly that success depends on the ease with which relatively young people can hit the ground running, as the appropriately American idiom has it. This in turn entails that the social function of senior and often distinguished researchers is to serve as a means by which young people can make a quick start. Newton acknowledged his debt to the giants on whose shoulders he stood; but the fabric of scholarship is such that even the shoulders of pygmies (relative to, say, Copernicus) are literally infinitely better than nothing. The first and the most important defence of basic research, in the United States and elsewhere, is that the process is cumulative. Not to have shoulders of any kind to stand on would spell some end-point for the research community which, for the whole of the past three decades, has been the chief source of intellectual innovation anywhere.

Unfortunately, the most immediate implications of this truth are that all basic research is equally valuable as a means of launching young and innovative people on the natural world. In present circumstances, that is probably as far from being the case in the United States as elsewhere. Indeed, the plurality of possible sources for the support of basic research of which the United States can still boast, and the freedom accorded by universities and other academic institutions to those who can recruit funds, ensures that even inward looking projects win an honourable place in the research enterprise. It would have been more persuasive, of Senator Baker but also of the United States Administration, if Dr Press's consensus document had squarely promised that the academic community would itself arrange that young people would always have durable shoulders on which to stand. Briefly, if the National Academy of Sciences wishes, as it should, to persuade the Administration to acknowledge the importance of continuity in basic science, it must also itself take on some responsibility for defining the circumstances in which basic research should be supported.

## Default

By default, the academy's consensus statement last week fell back on the weaker argument that industry would be robbed of the skilled people it needs if basic research is deprived of the funds to which it has grown accustomed. This line of argument is persuasive among academics. Unfortunately, neither the government nor industry, the supposed beneficiary, is likely to be impressed. Each of the sceptical but interested parties is fully aware that, for several years, the computer industry in the United States has been forced to manage without a sufficient supply of trained engineering graduates from the universities and that it has nevertheless managed creditably to look even Japanese competition in the face. Who would say that more support for basic science in the past decade would have remedied this state of affairs? And who would hold that academic institutions are necessarily the places at which the people best qualified to design

the next generation of integrated circuits, or the next elaboration of sophisticated software systems, should be trained? Most probably, even those who contributed to Dr Press's consensus document last week would judge this to be too pointed a question, too particular. The potential benefits of basic research, the riposte would go, by definition lie in fields that cannot as yet be defined. Yet Congress, the Administration and the throng of taxpayers and voters increasingly hag-ridden by inflation are understandably concerned with solving the unsolved problems that can be foreseen. Is it too much to ask that the basic research community should take time off from its usual preoccupation with the design of research-grant proposals to pay some attention to the preparation of young people for the modern world?

In such circumstances, the hope (still buoyant among even American academics) that industry will somehow step in to make good the deficiencies of federal spending is uncharacteristically unrealistic. Industry at present contributes only a small part of the cost of basic research carried out at universities and other research laboratories not directly controlled by government or by itself. For various reasons, not always calculations about the best way of paying less tax, corporations will no doubt seem more generous in the years ahead. (The public rhetoric will help.) Yet there is not the slightest hope that industry would be able — even if it were willing — to meet the full cost of reinvigorating engineering education in the United States. Moreover, there is the strongest reason why the basic research community, essentially the academic research community, should resist such a development — a university's chief responsibility is to its students and not to those who may eventually become its students' employers. It follows that the cost of university education, and of the research that keeps that education alive, should fall either on the students or on the community to which they belong. Third parties have no place. Paradoxically, then, the basic research community can best serve the needs of American industry by remaining as far as possible financially independent of it.

## Change

Thus it is that the basic research community is faced with a version of the dilemma with which Congress and the Administration are confronted. How, when the cost of carrying on without change is insupportable but when change as such is unacceptable, is the essence of an enterprise, or of an institution or even of a community (say, a nation-state) to be preserved intact? Congress is denying the Administration the budget cuts for which it asks because Congress cannot accept that its constituents' expectations of the modern world should be disappointed. The Administration is (so far, at least) inflexible on its defence budget because it dare not risk the frustration of its election promises. Both parties to this non-bargain know, however, that their failure to reach an accommodation is a recipe for disaster — one that would touch all sections of the community, not merely that concerned with basic research. The participants in Dr Press's colloquium might with advantage have taken hold of that point more directly. It is not merely their government's problem but theirs. Within their own parish, they should also have had the wit to recognize that among the chickens that have come home to roost are some born of academics' own firm belief that basic research is an occupational right (and that graduate students exist for the education of undergraduates); that the curriculum that has grown up in no more disciplined a way than Topsy is nevertheless sacrosanct; and that the time has come if not for change then for adaptation.

Fortunately, the American system of basic research is still so much more varied than any other that much of it is likely to survive the hard times ahead. The research community is right to protest at the wayward incidence of the latest round of budget cuts, and at the way in which they arbitrarily affect those projects that can most easily be cut. It would also be within its rights to complain that both the Administration and Congress have undervalued its contribution to the health, wealth and adaptability of the community in which it is embedded. But it cannot hope not to have change itself.



# Closure threat at FDA laboratories

## Drug testing facilities given low priority

### Washington

The Food and Drug Administration (FDA) seems to be playing cat and mouse with the 500 members of the staff of its drug research laboratories in south-east and south-west Washington. Last month, as the Reagan Administration's budget problems became apparent, the FDA management raised the prospect that its three drug investigation laboratories might be shut. At the same time, all members of staff were advised that they should ensure that their career record forms (SF171) were complete by the end of October. This is a familiar preparation for dismissal. Although the Senate Appropriations Committee has now decided that the emergency cut of \$40 million in FDA's budget should be trimmed by \$6 million, members of staff have the impression that the laboratories' days are numbered.

FDA's response to last month's budgetary problems seems to have been unusually panicky. Senior officials of the administration were given 24 hours in which to define a package of economies amounting to 20 per cent of the budget. At a meeting on 16 October in the auditorium of FDA's Humphrey Building, more than 200 scientists were told that the investigation had shown that the Bureau of Drugs could dispense with its divisions of drug biology and of drug chemistry and the biopharmaceutics laboratory. It had already been decided that the national centre for antibiotic analysis, supported chiefly by fees from manufacturers with products to test, will be disbanded, largely on the grounds that its work could be carried out by private laboratories.

Anxiety about the future of the laboratories stems from the form of words used at the meeting on 16 October by Dr Richard Terselic, deputy director of the Bureau of Drugs. Terselic is reported to have told his audience that on several occasions in the past two or three years, studies of the consequences of large budget cuts had repeatedly shown the laboratories to be dispensable. Asked what would happen if the cut required of FDA was more like 5 per cent than 20 per cent, he is reported to have said that "if you have decided that A is less important than B... there is a pressure in the system" that will make the less important activity vulnerable in future years.

A spokesman for FDA confirmed last week that the continuation of the work of the laboratories "has been identified as a low-priority area". He added that in the

light of the action by the Senate Appropriations Committee, it was not now likely that the laboratories would be closed during the present financial year (which began on 1 October).

FDA's drug investigation laboratories occupy a central role in its regulation of new drugs. Their functions include the checking of manufacturers' claims for the safety of new materials and an assessment of the efficacy of materials proposed for medicinal use. Among the laboratories' claims on public attention is that they were the source of the first warnings of the dangers of thalidomide in the late 1950s.

Members of the laboratories claim that

closure would compel the Bureau of Drugs to rely on post-marketing surveillance for the independent assessment of the efficacy of new drugs. It seems likely that such a departure from present practice would raise hackles in Congress. A letter-writing campaign by those concerned has been begun, while earlier this week it seemed likely that Congressman Albert Gore, chairman of the oversight subcommittee of the House of Representatives Committee on Science and Technology, would try to get to the bottom of FDA's intentions.

The outcome of a mandatory meeting of all the laboratory staff called for Tuesday this week is not yet apparent.

## UK universities plan to fire staff

British universities are still in a state of confusion over how to economize to meet the cuts in their grants. A major worry is still whether compensation to academics who lose their jobs will cost the universities more than savings in lost salaries. The answer will depend on how much extra money the government is willing to make available specifically for redundancy payments.

The Committee of Vice-Chancellors and Principals, which expects the first redundancies to be announced within a few weeks, now says that the matter is urgent. Last week it asked the University Grants Committee (UGC), which distributes government money among universities, to announce the terms under which it will compensate universities for redundancy payments.

The vice-chancellors' committee has provided the UGC with its own recommendations for the minimum compensation to which redundant academics should be entitled. Its scheme is intended to ensure fair treatment, especially for those academics whose contracts of employment do not give them security of tenure. Those with tenure, however, are likely to fight for higher compensation through the courts, making it still impossible to calculate the total redundancy bill. The best estimate remains that made in the summer of about £250 million.

The vice-chancellors' compensation scheme is based on that for civil servants. It is divided into three age categories — above 55 years, 50–55 years and below 50 years. Under the scheme, academics aged 55 years and over would be entitled to compensation broadly in line with the existing premature retirement scheme which provides a lump sum and an early pension. For example, an academic aged 55 years with 30 years' service and a salary of £15,410 would receive £23,115 as a lump sum and £7,705 index-linked annual pension. Total cost to the university would be £39,989.

The vice-chancellors say that existing

early retirement terms for academics aged 50–55 years are unfair. Accordingly, they recommend an additional lump sum payment to compensate for up to five years' loss of pension rights. Hence a redundant academic aged 50 with 25 years of service and an annual salary of £15,410 would receive a lump payment of £21,566 in addition to an index-linked pension of £6,742 a year and a further lump sum of £20,226. The total cost to the university would be £72,150.

Academics under 50 years of age would be entitled to a lump sum severance payment in addition to the normal lump sum and pension payable after retirement age. The total extra cost to the university would be only the lump sum payment. This would mean that an academic made redundant at age 40 years with 15 years of service and an annual salary of £11,425 would receive £23,800 on redundancy, a £6,426 lump sum and an annual pension of £2,142 after normal retirement age. The extra cost to the university would be £23,800.

The vice-chancellors' committee hopes that the UGC will adopt its scheme when deciding how to compensate universities for redundancy payments. But precisely how much money the UGC can make available remains uncertain. The £20 million already earmarked will almost certainly be inadequate, but much will depend on when the bills come in.

The vice-chancellors' scheme, however, is being given short shrift by the Association of University Teachers (AUT), the academics' trade union, which refuses to discuss the possibility of compulsory redundancy. The AUT believes that universities should make economies only through early retirement, frozen posts and non-payroll savings. It is adding its voice to requests that the government should spread the cuts over five instead of three years.

The AUT's threat to start legal proceedings at the first hint of compulsory redundancy, even before names are



named, has already had an effect. Pressure from the local union branch at the University of Aston in Birmingham persuaded the university to postpone compulsory redundancies at least until the next academic year. Local pressure also persuaded the University of Keele to withdraw its notification of 300 impending redundancies to the Department of Employment. Other universities have also decided to delay job losses in an attempt to avoid being the first to be dragged through the courts. A handful are also apparently planning not to balance their books for several years. Their policy of living on borrowed money perhaps for three or four years is favoured by the AUT.

**Judy Redfearn**

## Science research council

### Paying a price

The capacity of the Science and Engineering Research Council to make research grants to British academics is likely to be impaired, in the year immediately ahead, because of the over-spending by the council's Science Board, in the financial year that ended on 31 March. One of the ironies of this situation, described in the council's annual report for 1980-81, published last week, is that the chairman of the offending board, Professor John Kingman, has now succeeded Sir Geoffrey Allen as chairman of the council.



Kingman — poacher to gamekeeper?

According to the report, the council's allocation of funds to the science board will be reduced in the current year, while the board must repay to its master the outstanding amount of its over-spending.

The over-spending occurred, according to Professor Kingman, when the council decided to adopt a more bullish attitude to its spending to avoid falling into the trap of having money that it had not spent taken away by the government. In the event, it over-stepped the mark and received a sharp rap over the knuckles from the House of Commons Public Accounts Committee and a warning not to over-spend again.

Professor Kingman is confident that the council can do better this year. He expects spending to be within 0.15 per cent of the budget, the margin allowed by the Treasury, which he nevertheless complains is too narrow and would never be expected

in industry.

Although the science board over-spent by only a small fraction of its annual budget (£64.4 million in 1980-81), the effect on its activities has been marked. During 1980-81, the announcement of some research grants to universities had to be delayed for lack of funds. The repayments this year and next will also not help the board meet its future commitments, about one-third of which are to research and teaching in universities.

According to the annual report, support for university research, a third of which is channelled through the science board, had to be cut somewhat during 1980-81. Matters this year are complicated by the cut in university income which is increasing pressure on the council's research grants.

But the report, for which Sir Geoffrey Allen is responsible, says that spending on research grants should soon be restored at least to the level of recent years. Nevertheless, it is unlikely that the council will be able to support all grant applications given its highest merit grading of alpha.

The council, in 1980-82, spent £56.5 million on research grants out of its total budget of £207.8 million. A few more grants were awarded than in the previous year but the increase was due almost entirely to an unusually large number of requests for continuing support in some areas. A total of 1,983 research grants, valued at £74.5 million, were recommended in 1980-81 compared with 2,412 awarded in 1979-80.

**Judy Redfearn**

### New man in

Within a week of the resignation of the president and director-general of the French research agency, the Centre National de la Recherche Scientifique (CNRS) (*Nature* 5 November, p.3), a new director-general has been appointed: the long-time socialist party member M. Jean-Jacques Payan, a 46-year-old mathematician, and president of the University of Grenoble I.

The presidency of CNRS remains unfilled, although constitutionally the president has to help select the director-general. However, the director-general bears the main management load, and so the filling of this post was considered the more urgent.

M. Payan's first task will be to re-establish confidence in the CNRS management, to represent CNRS in the national colloquium on science and technology (which takes place in January) and to consider the reorganization of CNRS — particularly taking into account the strongly expressed wish of the engineers, technicians and administrators of the many CNRS laboratories to play a more significant role in policy-making.

**Robert Walgate**

## German energy policy

### The future unclear

Count Otto Lambsdorff, the West German economics minister, was balanced on a precarious political tight-rope last week, when he announced the governing coalition's energy policy for the next decade. Judging by his description of the "third continuation of the energy programme" (the second continuation was in 1977), he had little money, little energy and few plans. Or at least he had few plans he would admit to, for fear of rocking the coalition.

For example, to prepare the third continuation, Lambsdorff had asked several German economic institutes to make forecasts of energy needs and production. Usually a government will give guideline projections for the total economy in such circumstances; but Lambsdorff could not or would not provide them. And while the institutes clearly recommended a quadrupling of nuclear power generation by 1995 (from 3.7 per cent of primary energy to 17 per cent), Lambsdorff would only commit himself to the remark that "nuclear energy must provide an increasing percentage of baseload electricity".

Several factors lie behind this imprecision — strong local opposition within the Social Democratic Party (SDP), one of the coalition partners, to the expansion of nuclear power; the possible retirement of Chancellor Schmidt through ill-health before the 1984 general elections, complicated by the lack of an obvious successor; and the forthcoming fight in the local government elections next year, when SDP must present as united a front as it can.

Were it not for these political complications, the government would no doubt like to commit itself firmly to cheap nuclear power, which — behind the scenes — it sees as essential for maintaining the competitiveness of German industry. But if nuclear power is to be cheap, it must be plentiful, goes the calculation. A new German 1,300-MW pressurized water nuclear power station would cost nearly twice as much in Germany as in France, a German manufacturer claimed recently. According to the company, this is largely because the French companies were "tooled up" for long production runs, and environmental regulations and delays are less extreme in France. At best, nuclear power might contribute 25 per cent of German electricity by the mid-1980s, compared with France's 70 per cent by 1990, the company estimated.

Nevertheless, it is not good politics in SDP at present to be seen to be a strong supporter of nuclear power, in spite of the fact that there seems recently to have been a change of mood among the German public. Germany's strong dependence on oil and gas keeps domestic energy prices high, and the nuclear industry's claims that

nuclear power can be cheap is winning adherents.

Last week, however, Lambsdorff did commit himself to one change of policy. Like all Western ministers, he needs to save money during the world recession. Some of it will come from the coal industry — heavily subsidized in Germany to make its price competitive with the imported variety. These subsidies will be reduced, and cheaper coal imported. Germany should also shift away from burning "brown coal", and use it to provide chemical feedstocks. The missing energy would be provided by nuclear power.

●The German government would not be deflected from its commitment to a DM 10,000 million (£2,400 million) deal with the Soviet Union to provide Western Europe with natural gas through an overland pipeline from Siberia, Lambsdorff said last week. The deal would provide West Germany with around 12,000 million cubic metres a year, 30 per cent of its natural gas requirements. A large fraction of this gas will be used to produce electricity. Natural gas power stations at present produce 50 per cent more electricity in Germany than do nuclear stations.

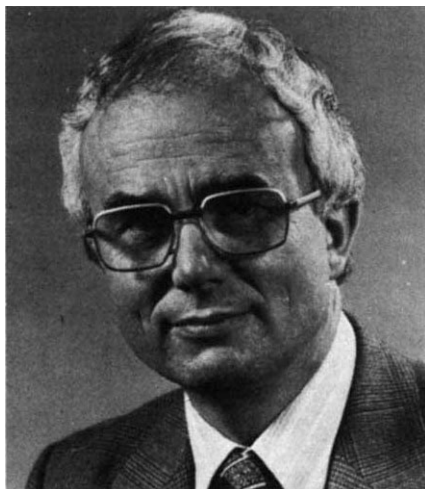
Robert Walgate

## UK Government Chemist New broom

The Laboratory of the Government Chemist, for long a respected but retiring establishment, may yet emerge from the shadows. For Dr Ronald Coleman, who will take over as Government Chemist when Dr Harold Egan retires at the end of December, intends that the laboratory should play a more central part in British science.

The laboratory, which employs about 400 people, provides a wide range of analytical services, mainly for the public sector. Thus it is concerned with the estimation of additives in foods and the characterization of oil-spill samples for correlation with suspected sources of pollution. Although supported by the Department of Industry, the laboratory is paid for specific services by government departments and local authorities. The laboratory has few dealings with the private sector and its research is largely that required to keep abreast of new analytical techniques.

Dr Coleman plans to change all this. He intends to broaden the remit of the laboratory, to make it "more open" and to



Coleman — talking change

forge links with both industry and the universities.

Dr Coleman would like the laboratory to "have a finger in more pies". In its analytical work he would like the laboratory to branch out into work on surface chemistry and on-line instrumentation. Mr Coleman himself has just been given responsibility for biotechnology in the Biotechnology Directorate set up last month, but the laboratory's role in this new venture remains to be determined.

Dr Coleman is also ambitiously planning coordinated programmes with industry, hoping that the laboratory could contribute technical expertise and industry the necessary funds. He has already talked to five interested industrial companies.

Dr Coleman points to his varied experience. At present he is deputy director of the National Physical Laboratory, but his career began with five years in the glass industry. His twenty-year spell with the United Kingdom Atomic Energy Authority involved work with nuclear energy, forensic science, geochemistry and biotechnology.

Isobel Collins

## Polish science

### Academy for change

Warsaw

Self-governance, the move towards "social control" of all sectors of public life, will undoubtedly bring considerable changes in the structure of Polish research planning. In particular, there have been vocal demands, coming especially from the scientists' lobby within the free trade union Solidarity, for parity of status between the universities and other higher educational institutions, the Academy of Sciences and its subsidiary institutes, and the institutes belonging to the production ministries (including the ministries of agriculture, fisheries and mining).

Funding tends to be less generous in the academy institutes than in those belonging to the ministries, many of which, it has been suggested, were founded more for reasons of prestige than for any real

research need. Although with Poland's newly-admitted unemployment problems — particularly among young graduates — closures are unlikely, a thorough-going reconsideration of the pattern of research is expected. Any moves from the top to start making changes would, however, run the risk of conflicting with the demand — expressed by the recent Solidarity congress — that scientists should have more say in choosing their own research topics.

Another Solidarity recommendation calls for the elimination of the barriers between the academy and the rest of the academic community. During the past few years the various academy institutes have become havens for young academics prevented from taking teaching posts for political reasons, and one of the changes suggested by Solidarity would be to allow scientists working at academy institutes to lecture to university students.

At the same time, there is considerable unrest both inside and outside Solidarity about the present anomalous status of the academy as a quasi-ministry, with its academic secretary responsible not to the academy but directly to the prime minister. A spirited debate, on this and other points, is expected when the general assembly of the academy meets in December, chiefly to debate the proposed new legislation on the academy.

A new bill on higher education is already on its way to becoming law, and (following the protests and strike-threats of last September) it now has the basic consent of the academic community.

Vera Rich

## Californian Medflies Brief armistice

Sacramento, California

Aerial spraying against the infestation of the Mediterranean fruit fly, now much curtailed for the duration of what Californians laughably call the winter, is likely to be resumed in earnest in the spring. But Mr Richard Rominger, director of the state Department of Food and Agriculture, told his Advisory Committee on Thursday last week that enough insect traps have been installed in the northern counties for next year's campaign to be effective.

Meanwhile, fruit farmers in Florida and Texas as well as California are more concerned with an unexpected consequence of the Californian campaign against the Medfly. The practice of fumigating fruit from affected areas of California with ethylene dibromide has raised environmental objections in Japan, one of the chief markets for United States fruit exports, and has also drawn attention to widespread use of ethylene dibromide for fumigating fruit from Florida and Texas (where other pests, such as the Mexican fruit fly, are common). Mr Rominger was hoping, last week, that the Japanese authorities would now accept as safe the Californian requirement that fumigated

## Asian mycotoxins

In the story "Yellow rain: Waiting for data", *Nature* 22 October p.598, it was mistakenly stated that a sample of vegetation had been taken from Laos for analysis. In fact it was taken from Kampuchea.



fruit should contain no more than 130 parts per billion of ethylene dibromide, but he had yet to persuade longshoremen in the United States that fumigated fruit could be handled safely.

Nobody knows the economic cost of the "abrupt interruption of the channels of trade" caused by the infestation and the consequent fumigation, but Mr Rominger thinks it exceeds by an order of magnitude the direct cost of aerial spraying to California, put at \$50 million for this year.

The reasons why California was caught napping by this year's events are now becoming clear. Operations against the Medfly are directed from the state's laboratory at Los Gatos, between San Jose and Santa Cruz to the south of San Francisco Bay. Medflies were first recognized in Southern California in June 1980, but that infestation was successfully dealt with by the release of sterile male flies, as had been the 1975 infestation near Los Angeles.

The importance of the nearly simultaneous outbreak in Santa Clara county (just north of Los Gatos) was appreciated only much later, partly because insect traps were more thinly spread but also because the complete life-cycle of the insect (22 days in tropical conditions) seems to vary enormously from one microclimate to another and may be as long as 77 days in the northern counties.

The density of traps in the northern counties at risk has now been increased to 50 per square mile, and adult Medflies are still sporadically being caught in them. Members of the laboratory's staff recall with anguish the early months of this year, when they were "desperate" for supplies of sterile males to release into the field. More than 10,000 million were used before aerial spraying was begun in mid-July.

The belief that supplies of supposedly sterile larvae from Peru were in reality fertile seems not to be supported by the evidence the laboratory has collected. The staff says that the Peruvian supplies of sterile flies were, however, poor in quality — some consignments yielded only 10 per cent of viable adult flies. The fertile females of Peruvian origin recognized in the field in June this year (on the basis of a fluorescent dye) may well have derived from larvae contaminating a Peruvian consignment after the bulk of it had been irradiated.

The present hiatus in the campaign against the Medfly has not stilled political recriminations, directed chiefly at Governor Edmund G. (Jerry) Brown. Mr Brown's stand against the aerial spraying of a carbohydrate bait laden with malathion, in the face of technical advice to the contrary, is widely resented by his opponents in the state legislature as well as by farmers' organizations and many agriculturalists.

Thus Mr Richard Niellson, a member of the State Senate, is concerned that the incident will have permanently damaged

California's reputation as a reliable supplier. He, among other members of the legislature, is planning to use the budgetary process now beginning to see that proper provision is made for the war against the Medfly in 1982 and succeeding years. But this year's experience, when the Red Cross centres set up to deal with casualties among the urban population of California were hardly used, has, Mr Niellson says, seen a decline of the "Rachel Carson syndrome" among the Californian electorate.

Mr Rominger, who with some of his technical advisers had threatened to resign if aerial spraying was not begun, hopes that the 17-member "pest prevention task force" due to hold its second meeting this Monday will insulate pest protection from politics. The committee is intended to present the governor and the state legislature with a general strategy for protection against all insect pests by the beginning of 1982. Meanwhile, the state will have to decide what to do about the 700 claims for civil damage which have been lodged, while inspection stations at the Californian border are likely to reappear.

For most Californians, the events of the past months are likely to prove cathartic. Next season, farmers (who are not prevented from ground spraying with malathion and more powerful insecticides) will take more active steps to protect their crops. The state as a whole seems to have recognized the economic importance of its agricultural industry, worth \$14,000 million a year. And people in urban areas have been left with a lasting impression of helicopter visitations at night (when the air is still), with their searchlights illuminating suburban gardens. "This is what it must have been like in Vietnam" said one whose white Volkswagen is still spattered with grey malathion bait.

## Nuclear Structure Facility

### Nylon slip shows

Water in the nylon insulating links of the charging system of the Nuclear Structure Facility, a 30 million volt tandem Van de Graaff accelerator for nuclear physicists under construction in the United Kingdom at Daresbury Laboratory in Cheshire, is now blamed for delays which will put the commissioning of the machine back at least to spring 1982.

A new material, Torlon, will have to be used in place of the monocast nylon in the pioneering machine, which is the highest voltage Van de Graaff yet designed. The nylon, Daresbury physicists found to their cost, contained a small proportion of water. This did not affect the insulating properties of the nylon at room temperature, but the conductivity rose a hundred-fold between 20°C and 35°C, the physicists discovered. So when the "laddertron" — a charging belt like a tank track made of alternating links of nylon and aluminium to carry charge up to the high-voltage

terminal — was first tested at full scale, and friction raised its temperature to 35°C, it failed.

Torlon, an American-made material, was not available when the accelerator was first designed, but in a test section of laddertron it has proved its superiority to the monocast nylon. The whole laddertron will now be rebuilt with Torlon.

However, the problems of the accelerator are not over yet. Unfortunately monocast nylon was used for other parts of the accelerator. Now the laddertron problems appear to have been solved, these too may have to be replaced.

Moreover, early next year the accelerator tube — the evacuated metal and ceramic tube down which the beam of nuclei must pass — will be installed and tested. And unless the Daresbury team are lucky for once, this too may cause delays. At Oak Ridge National Laboratory in the United States, problems with a similar tube set a 25 million volt machine back by a year — and even now the laboratory may have to be content with 20 million volts rather than the design figure.

Nevertheless the delays at Daresbury have had one bonus. All the experiments will be ready in time for the first beam, despite a low rate of funding which implied that only the bare minimum of experiments would have been possible earlier.

Robert Walgate

## Caltech

### Kellogg birthday

#### Pasadena

The Kellogg Radiation Laboratory at the California Institute of Technology combined its celebrations of its fiftieth anniversary last week with the dedication of a new custom built Megavolt accelerator and a seventieth birthday party for Professor William Fowler, the third director of the laboratory. Fowler, always ebullient, stole the show, but the laboratory was at pains to emphasize that it has been clever enough to equip itself with a new accelerator, albeit in the MeV range, when high-energy physics laboratories are wondering how long their GeV machines will be able to function.

The Kellogg Laboratory is the West Coast's chief monument to R. A. Millikan, the president of the California Institute of Technology when the laboratory was first formed. Millikan's original prospectus included the use of high-energy radiation in the treatment of cancer. During the Second World War, the laboratory played an important part in the determination of nuclear cross-sections needed for the Manhattan Project. Since then, it has been principally concerned with the application of nuclear physics to other fields, astrophysics in particular.

The new accelerator, represented last week by its external yellow tank and christened as if it were a sea-going object,

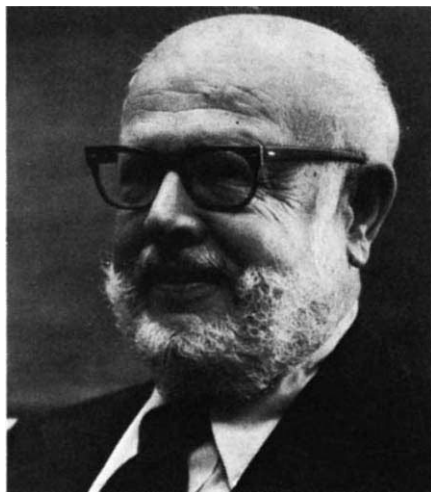


has been designed for high ion current and voltage stability. It will be used, among other things, for the laboratory measurement of nuclear cross-sections relevant to the processes of nucleogenesis which are at present uncertain.

But Dr Barbara H. Cooper also outlined a scheme for using the accelerator as a charge spectrometer for checking the claim by William Firkbank and his colleagues at Stanford University that electric charges exist which are a fraction of the electronic charge, and which might represent free quarks. Preliminary estimates suggest that by using an argon beam for sputtering free atoms from solid surfaces, it should be possible to evaporate one of the small niobium spheres used in the Firkbank experiments in about an hour, and that the tandem accelerator should be capable of detecting one fractionally charged particle in as many as  $10^{18}$  niobium atoms.

While astrophysical problems will remain a central part of the Kellogg Laboratory's programme, diversification is also in the air. This is the spirit in which the laboratory is involved with studies of the release of radon-222 as possible predictors of major earthquakes, just now a matter of public interest in California.

According to Professor Thomas A. Tombrello, two of the network of radon monitors maintained by the laboratory near the San Andreas Fault had revealed a substantial increase of radon evolution since the early weeks of August. The



W.A. Fowler at seventy.

increased rate of evolution (which continues) has been closely correlated in time even though the two instruments are separated by 100 kilometres, and is said to resemble that which preceded the 1979 earthquake of magnitude 6.7. That event was marked by a transition from the normal compressive stress across the surface rocks of the San Andreas Fault to an extension — thought to stimulate radon release — but also by a cessation of radon release in the days immediately before the earthquake.

Professor Tombrello prudently avoided prediction last week, saying only that "sometimes, perhaps in 25 or 50 years, one

of these events is going to be a precursor of a major earthquake". He complained, however, that financial support for earthquake studies of this kind in California was less than that in China and Japan. "Since 10 million people would be affected by a major earthquake, it would be worth putting a little money into it."

## Albanian development

### Hoxha looks ahead

Albania, too, has an urgent need of science and technology, according to party leader Enver Hoxha's speech to the congress of the Albanian Workers' Party last week. But although the "deepening of the technical scientific revolution" has encouraged teaching and research, practice lags behind precept. Mr Hoxha said that the most obvious need is for a mechanism for the gradual transfer of innovation on a "wider front" aimed at the "radical" transformation of technology and production.

Mr Hoxha's criticism came in a speech praising the achievements of Albania's system of education, scientific education in particular. He drew particular attention to Albanian achievements in hydroelectric and railway engineering, the sinking of deep wells, geological prospecting, stock-breeding and the machine tool industry. He emphasized that these had been accomplished by the Albanian people "relying completely on their own forces" — a justification of the country's long standing isolation policy. Although the Academy of Sciences is still the major centre for scientific research in Albania, Mr Hoxha said that the Committee for Science and Technology set up this year was an important instrument for the "better direction and organization" of science.

The targets for the Albanian 1981–85 five-year plan are more ambitious than in any previous quinquennium. Industry will receive some 46 per cent of the total investment budget, with special emphasis on mining and energy resources. Oil extraction is expected to rise by some 60 per cent, coal by 48 per cent and that of proved mineral resources (chromium, copper and iron nickel) from 30 to 200 per cent. The chemical industry will be considerably expanded, with an expected rise in production of some 65 per cent by 1985.

Mr Hoxha was at pains to say that these targets are "scientifically based and fully achievable". Furthermore, to provide the necessary personnel, special attention will be given to higher education. Student admissions during the next five years will rise by 45 per cent, new courses will be introduced and there will be a considerable expansion of postgraduate courses. Publication of "political, scientific, technical and artistic" books will rise by one million copies over the previous quinquennium and educational cinema, radio and television will be expanded. **Vera Rich**

## Hoyle on evolution

The serious part of the Kellogg symposium provided Sir Fred Hoyle with an opportunity for a moderate (and self-critical) statement of his case for disbelieving conventional views about the evolution of the Universe, the "big bang" among them. Hoyle has been associated with the Kellogg laboratory since his collaboration in the mid-1950s with W.A. Fowler and the two Burbidges (Margaret and Geoffrey), now known as the gang of four, on the problem of nucleogenesis.

Hoyle said last week that, although content in the mid-1960s to give the supposed connection between the microwave background radiation and the big bang a "good run for its money", he had now lost patience with this approach. Two of his reasons involve the origin of life — the calculated time since the origin of the Universe of 10,000 million years or so is not enough to account for the evolution of living forms, while adiabatic expansion of the Universe would have been inimical to the evolution of highly ordered forms. But Hoyle also said that new evidence in support of the big-bang hypothesis was emerging only slowly. Yet "when people are on the right track, new facts emerge quickly". Hoyle said

he would change his view if it turned out that neutrinos have a mass of between 20 and 30 electron volts.

The essence of his argument last week was that the information content of the higher forms of life is represented by the number  $10^{40,000}$  — representing the specificity with which some 2,000 genes, each of which might be chosen from  $10^{20}$  nucleotide sequences of the appropriate length, might be defined. Evolutionary processes would, Hoyle said, require several Hubble times to yield such a result. The chance that higher life forms might have emerged in this way is comparable with the chance that "a tornado sweeping through a junk-yard might assemble a Boeing 747 from the materials therein".

Hoyle acknowledged that steady-state theories of cosmologies, of which he was one of the chief exponents in the 1950s, are not now tenable because of the evidence for evolutionary galactic and stellar processes. But the big-bang view is similarly not tenable because of the way in which it implies the degradation of information. Of adherents of biological evolution, Hoyle said he was at a loss to understand "biologists' widespread compulsion to deny what seems to me to be obvious".

# CORRESPONDENCE

## Good as gold

SIR — In the current debate on a gold standard the stumbling block is the fundamentally wrong premise in the quest to fix a price. It is not possible to fix a price for gold in terms of any existing currency. Far better to consider not a price but a value. Gold has a value as a medium of exchange, in other words as a currency. Consider the implications of using gold as money.

Assume all gold reserves minted into coins of standard weight and fineness. At a given date equate the number of coins so available with the figures used internationally in representing the price of goods and services in circulation. One coin will be found to equate to many hundreds of dollars.

Now start using only gold coins to pay for goods and services. Note the dramatic fall in the numbers on the price tickets (the first skirmish in the war on inflation). Continue to mint all newly-won gold into the standard coins. Charge the weight of gold going into industry or jewellery at the value of the equivalent number of coins. Paychecks would of course come down accordingly. Cheques and all paper transactions could be continued, but only against deposits in the equivalent number of coins. Gold money thus on deposit would pay interest whereas gold now in the bank does not.

The result would be a stable currency and stability of values. The gold-producing countries would not get any richer (you can't eat gold) whereas countries with exports of commodities or manufactures, instead of suffering from "lack of hard currencies" would find their products are as good as gold.

This desirable state of affairs would continue as long as the supply of coins was adequate to satisfy the needs of international commerce. However, the rate of increase of the net worth of the world is historically greater than the rate of increase of gold production and is likely to remain so. In this situation the remedy would be to have a periodic revaluation of the value of the coin. Clearly this value is going to tend upwards and, as a concomitant, the numbers on the price tickets will go down — the very reverse of inflation. Gold thus produces capital gains.

The return to gold usage (rather than a gold standard) would provide a solution to currency and exchange problems and, since the money supply would be finite, a cure for inflation and a clear directive for monetary policy.

JOHN H. HARRIS

New York, USA

## Harsh words

SIR — Your comments on Dr Rupert Sheldrake's book *A New Science of Life* (*Nature* 24 September, p.245) are very harsh. One can hardly pretend that our current understanding of development and its genetic control is adequate. We should not even believe that it is oriented in the right direction. The concept of morphogenetic fields is neither unscientific nor without support from some distinguished embryologists. If the same concept is extended to all aggregations of matter, animate or otherwise, it may be considered as a bold, hazardous, foolhardy or

unwarranted generalization, depending on one's attitude towards new thought. Just a hundred years ago if somebody suggested that the sequence of nucleotides in DNA constitutes the information required to make a fly, a frog or a man from a small bit of cytoplasm it would have been dismissed as preposterous. If there is no obvious way of testing experimentally Dr Sheldrake's hypothesis of formative causation, it does not preclude its possibility in the future.

Many embryological theories depend on recourse to morphogenetic fields in spite of the recent progress in genetics and molecular biology. No real progress can be made in understanding the importance of these fields without identifying the nature of the variables along the gradients. Perhaps the only measurable variable to which the origin of a gradient can be traced is the concentration of diffusing substances. Positional information which adds a new dimension to the concentration of diffusing substances is also now accepted as a fundamental mechanism of morphogenesis. However, even these powerful theoretical tools have not successfully accounted for any single embryological event. In fact it would be preposterous to assume that development of complex organisms can be explained solely on the basis of the current theoretical platform on which molecular biology stands. Recourse to new conceptual framework and unconventional mechanisms is imperative to understanding complex biological processes such as morphogenesis.

K. VASUDEVA RAO

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## Page charges

SIR — Frank Close's review of high-energy physics journals in *Nature* 1 October (p.369) reveals a misconception that may be widespread enough to warrant correction in public. I refer to his statement that "in times of financial stringency European authors are often unable to publish there [in *Physical Review*] as a result of page charges". The fact is that the page charge system, not only in *Physical Review* (including *Physical Review Letters*) but in all journals published by the American Institute of Physics, is a voluntary one. Payment of page charges is requested, but acceptance of a contribution is not conditioned by whether page charges are paid. The only effect of nonpayment is a delay in publication; and even that is not imposed on papers in *Physical Review Letters*, or on the newly instituted rapid communications section in *Physical Review*.

I hope I will not be misconstrued as urging authors to submit papers to our journals and not honour the page charges when they are in a position to pay them. I do not even mean to solicit papers for the journals. Those costs that are not met by page charges have to be made up from higher subscription costs for persons and institutions that are not members of the various societies; and we are as concerned as anyone else over the effects of ever higher costs to libraries. I do feel, however, that it is important that the true state of affairs be completely understood.

GEORGE L. TRIGG  
(Editor)

*Physical Review Letters*,  
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## Belgium's power

SIR — Your recent article on nuclear safety in Belgium (*Nature* 24 September, p.249) prompts me to explain some points about Belgium's approach to nuclear power.

First, the nuclear power station licensing procedure in Belgium involves two stages: (1) on approval of all planning and logistical aspects and the safety guidelines, permission is granted for construction to start and (2) after subsequent safety analysis and approval, permission is granted to put the power station into operation.

During plant construction there is continued liaison between the public authorities, technical experts, suppliers and the station owner, to ensure that all safety aspects are in line with the Belgian regulations. This process involves the exchange of about 2,000 questions and answers, and has the advantage that the most modern safety innovations are incorporated into Belgian nuclear power stations at an early stage. This could clearly not happen if the design had to be frozen at the time of the construction permit.

Given the long construction period required for such plants, this procedure eliminates the problem of premature technological obsolescence. It is also interesting to note that Belgium is the only country to submit nuclear power station designs for approval to a committee of international (EEC) experts.

Second, the question of the number of specialists officially appointed to deal with nuclear safety was somewhat misrepresented in your article. Belgian law confers supervisory authority for the nuclear sector to a state-approved inspection organization, Vinçotte. Other official government bodies, however, exercise ultimate control in this field. So in fact there are many more than the 17 Vincotte inspectors involved in supervising the safety of the Belgian nuclear power stations.

On the other hand, although it is true that the International Atomic Energy Agency has cited the figure of 50 experts as a standard force to deal with nuclear safety in a country like Belgium, the responsibilities of such a force would include operational control, regulatory missions, evacuation plans and so on, in addition to overseeing general safety precautions. In Belgium these aspects are not neglected, but are properly dealt with by the governmental bodies and technical experts mentioned above, (including the "Commission for questions concerning radiation").

Finally, with respect to the threatened strike by the employees of Vinçotte, their qualms did not stem from questions of power plant operations, but rather from the uncertainty of their career prospects.

So to summarize, (1) the procedure followed by the utilities before putting a nuclear power plant in operation strictly follows Belgian law — the strictest in the world, according to IAEA. And (2) the force of experts and inspectors in charge of safety supervision is perfectly adequate, both in number and competence, and is in a position to enforce the nuclear safety regulations, as it has indeed done (nuclear power stations have been operating in Belgium for 20 years).

R. VAN DEN DAMME

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## NEWS AND VIEWS

## High energy physics grows tenfold

from Robert Walgate

THE first ever controlled collisions of fundamental particles at centre of mass energies of over 540 GeV — ten times higher than previously achieved, and an energy at which spectacular physics is expected — are beginning to be observed at the new proton antiproton collider at the European centre for nuclear research (CERN), even before the machine is out of the hands of its constructors.

"We take data while the engineers take their coffee breaks", Carlo Rubbia, leader of one of the two experiments now in place, said last week. Rubbia — a well-loved Italian-American maverick of high-energy physics — in fact proposed the whole idea of the collider, in which bunches of protons at 270 GeV collide with oppositely moving bunches of antiprotons. The proposal came in, in a report at the 1976 Aachen neutrino conference titled "Looking for the intermediate vector boson with existing accelerators". He has not been slow to reap the rewards now the machine is in place. So far his experiment (UA1) has recorded some 20,000 events, but no intermediate vector boson.

On the other side of the collider (which is in fact the super proton synchrotron modified to take colliding beams) is experiment UA5, headed by Dr John Rushbrooke of the University of Cambridge with professors Klaus Bockmann of Bonn, Jacques Lemonne of Brussels and Gösta Eksborg of Stockholm and their groups. UA5 has taken 1,500 pictures (one of which appears below) with another 10,000 expected in the next month.

The events of UA1 and UA5 together are only the first scratching of the surface of physics at these exalted energies, but

already there are some indications of what may or may not be expected.

The physics needs to be seen in relation to two quite separate classes of prediction. On the one hand, there are some peculiar data from cosmic ray experiments — a handful of "Centrauro" events and others — which indicate that there may be a substantial change in physics at energies close to, or perhaps somewhat above, the energies of the new collider<sup>1</sup>. On the other, there are the predictions of the Salam-Weinberg unified gauge theory of the weak and electromagnetic interactions, which has been well-tested at lower energies in electron and neutrino scattering experiments.

This theory is the backbone of most current attempts at a "grand unification" of all the forces, and it predicts the existence of three intermediate vector bosons ( $Z^0$ ,  $W^+$ , and  $W^-$ ) at masses of around 80 GeV. These should be created at collider energies, and should be seen to decay as predicted by the theory. The theory also predicts new scalar particles called Higgs' particles, but the masses of these are, generally speaking, undetermined and the particles may or may not turn up in collider experiments.

So far there are no data from CERN on the intermediate vector bosons, but that is not unexpected. Estimates suggest that the IVBs will only be produced in small numbers, and too few events have been collected so far to expect one among them. Finding the bosons also depends on increasing the luminosity of the collider (effectively the number of collision per second per unit interaction cross section) by orders of magnitude beyond those presently being reached at CERN. Design luminosity was  $10^{30} \text{ s}^{-1} \text{ cm}^{-2}$ ; the present achievement is  $2 \times 10^{25}$ . A luminosity of  $10^{29}$  would give Rubbia about 10 Ws and

1–2  $Z^0$  a day, he estimates, allowing for his detector efficiency.

CERN machine physicists have however not yet attempted to "tune" the collider to give its maximum luminosity. Two effectively independent factors of 50 could be obtained relatively easily, it is believed, which would bring the machine within a factor of 2 of a luminosity of  $10^{29}$ . The remaining factor of 2 might be made up within a year, machine physicists feel, with  $10^{30}$  remaining so far a very distant goal.

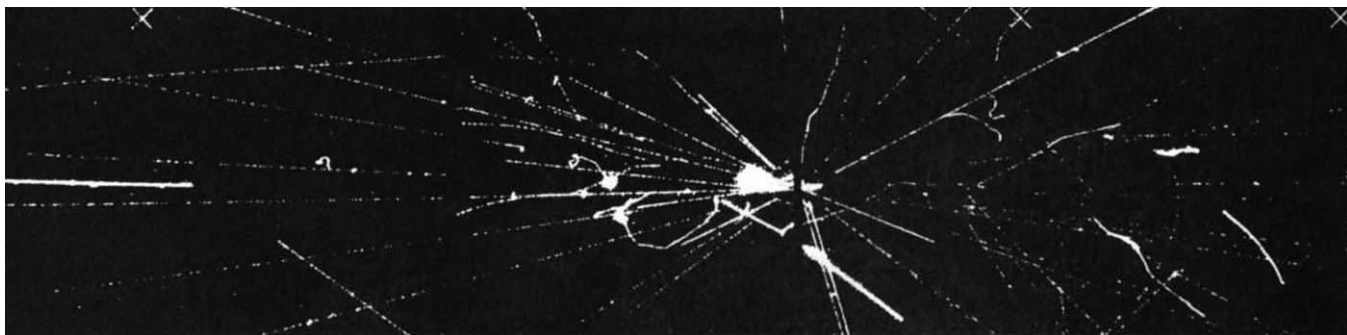
More easy to check are the cosmic ray results. These indicate that some extraordinary and inexplicable interactions may set in at centre of mass energies of the order of 1,000 GeV (This is around twice the collider energy, but the cosmic ray experiments have a detector threshold at about that level, Rushbrooke believes, so the physical threshold may be lower.) These Centauro events — of which only five or so have been observed, cosmic ray intensities being so low at this energy — show the production of very large numbers of charged particles (track multiplicity) and a mysterious near absence of neutral pions. Also the average transverse momentum of these and other high energy cosmic ray events rises with multiplicity, something quite unknown in lower energy data.

However, so far neither UA1 or UA5 have found any clear indication of these phenomena at the collider — though exact results await more precise data analysis. Confirmation of the cosmic ray results may have to wait for higher statistics — or for a collider of even higher energy, such as the 2,000 GeV centre of the mass energy collider being planned at Fermilab near Chicago for action in 1984 or 1985 (Ronald Reagan willing). □

Robert Walgate is Chief European Correspondent of Nature.

1. Latters, C.M.G., Fujimoto, Y., & Hasegawa, S. *Physics Reports* 65, 3 (1980).

One of the first 540 GeV p-p events to be recorded in the Cambridge-Bonn-Brussels-Stockholm streamer chamber (experiment UA5), showing clear forward and backward cones of tracks. The streamer chamber is the only detector on the collider to record particle tracks clearly close to the interaction point. It will provide a first survey of the physics of interactions at these new energies.



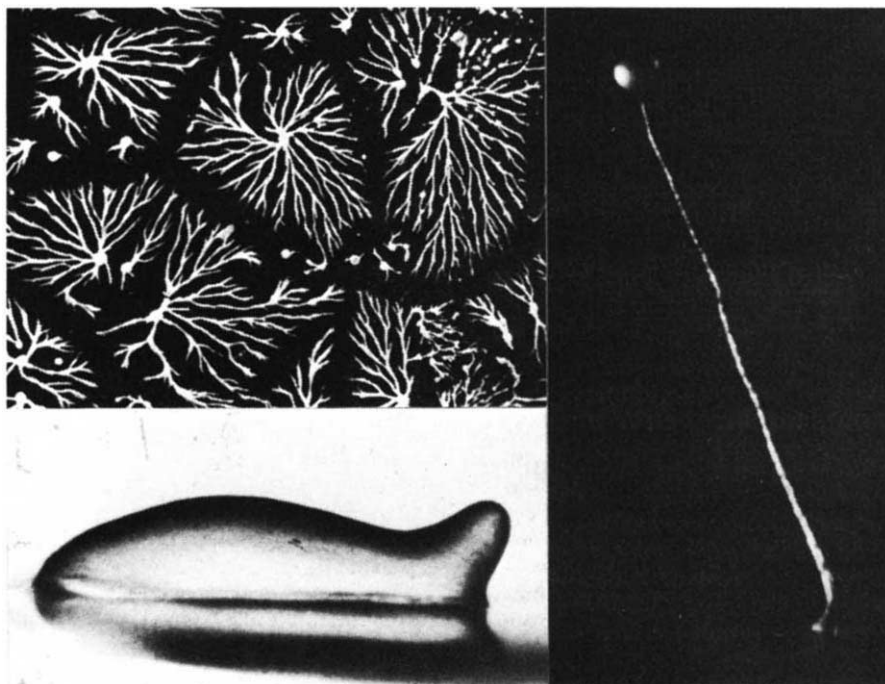
# How cells live together

from Robert Kay

THE cells of a higher organism normally behave in a completely social way; it is the occasional deviant — such as a cancer cell — that attracts our attention. But how is cooperation between cells in a multicellular organism established? The problem is clearly seen in the cellular slime moulds, such as *Dictyostelium discoideum*. They feed as free-living amoebae, but when starved the cells aggregate by chemotaxis (to cyclic AMP) forming a small multicellular organism. This 'slug' though without the benefit of nerves or sense organs, can orientate and move towards light or heat. Eventually it differentiates into a fruit, with a stalk supporting a mass of spores. It was apparent from a recent meeting\* that to establish multicellularity cell-to-cell adhesion (and production of a sheath around the organism) and communication systems to control both movement and differentiation are necessary.

Cohesion is thought to be brought about by any of four systems in *Dictyostelium*: through contact sites A and B (cs A,B), by the discoidin lectins and by a 95K membrane protein from slug cells<sup>1</sup>. Appropriate antibodies and treatments can block cohesion via each system and now the pressing problem is to establish their respective roles *in vivo*. Progress is being made using cohesion-defective mutants. In a ts mutant described by Sussman (University of Pittsburg) cohesion during aggregation is normal at the restrictive temperature but is lost abruptly at the time slugs would form in the wild type. This indicates that the aggregative cohesion systems are replaced by a new mechanism in the slug. As the 95K protein does not appear at the restrictive temperature, it probably is part of the new mechanism; analysis of wild-type membrane extracts that restore cellular cohesion to mutant cells should test this supposition. Mutants affecting cs B, on the other hand, develop quite normally showing that cs B has only a minor role in developmental cohesion (Vogel, Max-Planck-Institut, Munich); its major function is probably in binding bacteria preparatory to phagocytosis and in cell-to-substratum adhesion. Similarly, in a related slime mould species cohesion is unaffected by mutation of its lectin (Francis, University of Delaware), so that by elimination, cs A is probably responsible for aggregative cohesion.

The signal coordinating forward movement of cells in a slug may or may not



After a few hours of starvation *Dictyostelium* amoebae initiate and relay cyclic AMP signals which guide them into streams leading to collecting points, where the cells mound up (top left, in dark field). These mounds transform into migrating slugs, each up to about 2mm long (lower left). The slug has an anterior organising 'tip' and eventually it differentiates into a fruit consisting of a mass of refractile spores supported by a cellular stalk (right).

be cyclic AMP, but the steering of the slug towards light involves something new. In a model proposed by K. Williams (Max-Planck-Institut, Munich) differential illumination of the slug tip generates a transverse gradient of an extracellular signal molecule, slug turning factor (STF), in the slug, to which cells respond by a change in direction, most likely by chemotaxis. The properties of STF are consistent with this model: it is made in response to light, high environmental levels disorientate phototaxing slugs and it is itself a slug chemotactic agent. Interestingly, STF, the stalk cell differentiation inducing factor DIF (Kay, ICRF, London) and an inducer of fruiting<sup>2</sup> are all low molecular weight, hexane-soluble factors; whether these similarities point to identity or close relationship is as yet unknown.

Cyclic AMP signalling controls both cell movement and gene expression (for discoidin, at the level of transcription; J. Williams, ICRF, London) and it is becoming apparent that the coupling machinery used is similar to that of some mammalian hormones. Thus, *Dictyostelium* adenyl cyclase has a GTP binding and cholera-toxin-sensitive component analogous to the mammalian G protein (Leichtling and Rickenberg, National Jewish Hospital, Denver), whilst the major and most specific cytoplasmic cyclic AMP

binding protein (of about molecular weight 40,000; Veron, Pasteur Institute; Cooper, University of Michigan; van Driel, University of Amsterdam) inhibits the catalytic subunit of mammalian cyclic AMP-dependent protein kinase (Rickenberg) indicating that it too is part of a cyclic AMP-dependent kinase, as reported some years ago<sup>3</sup>. Similarly the  $Ca^{2+}$ -mediated effects of cyclic AMP signalling on myosin phosphorylation involves calmodulin (Moruta, Max-Planck-Institut, Munich).

It has long been suspected that coordinately regulated genes (such as those regulated by cyclic AMP) might be distinguished by a common control element (see, for example, ref. 4). Just such an element may now have been found (Lodish, MIT). It is a sequence 300–500 bases long, interspersed in the genome and transcribed into 50 or more messenger RNA species by the fifth hour of development, but absent from vegetative RNA. Different sequences mark other groups of mRNAs present later in development and in growing cells<sup>5</sup>. By a fortunate coincidence a powerful means for finding the function of these sequences is now at hand. Firtel (La Jolla) described the first efficient transforming plasmid in *Dictyostelium* which has a selectable marker with a suitable *Dictyostelium* promoter and origin of DNA replication,

Robert Kay is a member of the scientific staff of the Imperial Cancer Research Fund, Mill Hill Laboratories, London, UK.

\*The conference on Gene Expression and Membrane Changes in Cellular Slime Molds was organized by Günther Gerisch and held at Tübingen, September 2–6, 1981 with support from the European Molecular Biology Organization and the Max-Planck-Gesellschaft.



and can give transformation frequencies as high as 1 in  $10^4$  cells. It should now be possible to see what happens to a foreign gene introduced into the cell attached to one of the above putative control sequences. In principle it should also be simple to isolate genes essential for development by complementing developmental mutants with wild-type DNA inserted into the plasmid.

The *Dictyostelium* slug is a sort of free-living embryonic field and there is great interest in how a regulative pattern of pre-stalk and pre-spore cells is established in it. The problem divides into two: what induces an amoeba to differentiate into a pre-stalk or a pre-spore cell and then what establishes the spatial pattern of cell types? From work with isolated cells it appears that cyclic AMP is required for both stalk and spore differentiation but that DIF can, by favouring stalk formation, control the

choice of pathway (Kay). Several groups (Takeuchi, University of Kyoto; David, Munich University; Durston, University of Utrecht; MacWilliams, University of Massachusetts) strongly felt that sorting out of pre-stalk and pre-spore cells is an important way in which patterning is established (see ref. 6). However, we are a long way from knowing if this is the major or only way to generate pattern, especially as during regulation of pre-stalk isolates, and in *D. mucoroides* slug migration (where a stalk is continuously laid down), cells seem to differentiate according to their position (Gregg, University of Florida).

It is evident that even the lowly multicellularity of *Dictyostelium* is complicated enough for our present comfort. It would be nice to know that the tricks learnt by aggregating slime mould amoebae are still used (along with new ones) by higher and less tractable organisms. Of course, until both are understood this remains debatable, but recent evidence that many mammalian hormones<sup>7</sup> and their response mechanisms are also present in simple eukaryotes suggests that tricks once learnt are not forgotten. □

Unfortunately, in an amorphous specimen orientation is random and so unspecified, and even in a crystalline specimen the outgoing signal cannot be directed with any precision. However, the near-edge data involve rather slow and easily scattered electrons which will scatter between several atoms before eventually returning to the centre. Having travelled more widely than single-scattered electrons their phase contains information about the three-dimensional disposition of the neighbours, but is much more difficult to interpret.

The limitation of information to radial structure, that is, to the radial distribution function, is a general property of weak scattering probes such as X-rays, neutrons or electron microscopes. It is particularly restrictive in systems where a knowledge of bond angles as well as bond lengths is important to an understanding of the structure.

Having proved the technique for well characterized materials the Daresbury scientists can be expected to tackle more exotic systems such as multi-component glasses or liquids, or biological molecules for which structural information is not available in advance.

The X-ray absorption experiments which have been first past the post in the race to publish are in fact only a part of the activities planned by university and industrial groups using Daresbury. In the first instance two beams of radiation are available. One provides for X-ray topography, and for X-ray interferometry, as well as for the absorption experiments referred to above. The other, providing radiation at longer wavelengths in the vacuum ultraviolet and soft X-ray range has two main experiments. The first VUV station to be operational measures the photoemission of electrons from surfaces and has the capability of resolving the direction as well as the energy of the escaping electrons. The second experiment measures the absorption cross sections of atoms adsorbed on surfaces and provides information in the surface context very similar to the structural information available for X-ray absorption experiments performed on both samples. Both these experiments will further our understanding of surface structure — important in catalysts, semiconductor technology and other areas of surface science.

In the near future more beam lines will become available, one providing for experiments in the area of atomic and molecular physics. Another will make available radiation in the infrared region for experiments on large organic molecules thus extending even further the range of wavelengths and types of application of the facility.

D.J. Thompson's team at Daresbury is currently completing the final stages of fine tuning of the SRS which now operates to specification with a regular schedule of users. Soon a major enhancement of the source will be made with the installation of

1. Steinemann, C. & Parish, R.W. *Nature* **286**, 621 (1980).
2. Sussman, M., Schindler, J. & Kim, H. *Exptl Cell Res.* **116**, 217 (1978).
3. Sampson, J. *Cell* **11**, 173 (1977).
4. Britten, R.J. & Davidson, E.H. *Science* **165**, 349 (1969).
5. Kimmel, A.R. & Firtel, R.A. *Cell* **16**, 787 (1979).
6. *Nature* **291**, 532 (1981).
7. Le Roith, D., Shiloach, J., Roth, J. & Lesniak, M.A. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6184 (1980).

## Tripping the light fantastic

from John B. Pendry

THIS issue of *Nature* contains the first data taken on the new Synchrotron Radiation Source (SRS) housed at the Daresbury Laboratory. The source is a 2 GeV electron storage ring, with a design current of 370 mA, dedicated to the production of synchrotron radiation. Scientists at British universities and laboratories now have access to their own central facility for synchrotron radiation which will enable them to compete on equal terms with scientists in other countries.

The paper, by Greaves, Durham, Diakin and Quinn, (see p. 139) is an extension of the now familiar technique of using X-ray absorption fine structure to determine local crystallography [Sayer *et al. Phys. Rev. Lett.* **27**, 1204, 1971]. The principle is that X-rays eject an electron from the inner core of an atom. The X-rays can be tuned to a particular type of atom because the inner shell ionization energies are strong functions of atomic number. When the ejected electron encounters the atomic neighbours of the excited atom it can be scattered and some of these secondary waves get back to the central atom. There, because the electron is described by a wave function, an interference process takes

place that may suppress or enhance the absorption cross section according to the phase of the scattered wave. Increasing the X-ray energy changes the electron wavelength and modulates the phase in a way that depends on the distance of the scatterers from the central atom. In this way X-ray physicists have discovered how to make a microscopic radar station. So far we are on well-trodden ground. What is new about the Daresbury paper? The experiments reported are X-ray absorption measurements on copper and manganese and the message is not so much an unravelling of the spectra of these materials as a *jeu d'esprit* of experimental and theoretical excellence demonstrating that these spectra can be easily and accurately measured and, what is more, interpreted in a region of the spectrum, the near-edge region, which has previously been regarded as too complex to provide useful information.

The ability to interpret near-edge data goes some way towards removing a restrictive aspect of the X-ray absorption technique. For a radar station to give complete information about surrounding objects the precise orientation of the aerial must be known when the signal is sent out. Otherwise what emerges is merely a radial map with no directional information.

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a powerful superconducting magnet (called a "wiggler" for technical reasons) which will accelerate the electrons in the source so strongly that the spectrum of radiation, currently cutting off at a wavelength around 0.3 Å, will extend down to 0.1 Å.

Although synchrotron radiation sources will soon be widely available in numerous countries, the technique still shows massive potential for further development. The Daresbury ring at full power produces several tens of kilowatts of raw radiation, enough to destroy most experimental specimens. Yet, because the requirement is mainly for spectrally pure radiation, most

of the power is dissipated as heat in a monochromator. If by some means the power available could be channelled into radiation of one wavelength a new and even more extraordinary generation of experiments would become possible, investigating nonlinear properties of matter. Perhaps the most radical proposal is to combine the power of synchrotron radiation with the coherence properties of a laser and produce the so-called free-electron-laser. It is gratifying to see UK scientists involved in all phases of the development and exploitation of synchrotron radiation. □

produces two major tRNA<sup>Tyr</sup> species one of which has the first position of its anticodon occupied by the modified Q base. The one with the unmodified anticodon, GUA, but not the other, is capable of acting as a suppressor to the amber termination codon UAG; in other words it can recognize UAG and insert tyrosine so preventing termination of translation. Again we have a wobble anomaly in that the interaction in the third position is between two G residues. The discovery of this naturally occurring suppressor in *Drosophila* was aided by an interesting assay system for breakthrough or suppression of a chain termination signal. This involved injecting into a *Xenopus* oocyte some of the tRNA to be assayed and some TMV RNA. If suppression occurred, this resulted in the formation of a 160,000 molecular weight protein, identifiable by polyacrylamide gel electrophoresis. Thus Bienz and Kubli also showed that wild type tRNA<sup>Tyr</sup> from yeast (with no Q base) would also act as suppressor in this system. They suppose that the presence of the Q base somehow inhibits the misreading of the UAG codons involved in this type of suppression.

Finally Bossi and Roth<sup>4</sup> report on a different sort of suppressor effective in the suppression of the effect of a frameshift mutation. A frameshift mutant occurs when an extra base pair is inserted or a normal one left out during the process of replication. In either case the reading of the bases constituting the codons gets out of phase when the site of the mutation is encountered during translation. This results thereafter in a garbled polypeptide. Such a garbling of the message due to an insertion could be corrected if a tRNA was available which read four bases instead of three at a site near to the original mutation. Several mutant tRNA species can be produced which have this effect. Most of them respond to a region of the messenger RNA where there is a sequence of four identical bases; reading of the fourth base, so to speak, takes up the extra inserted base. The mechanism by which this occurs is obscure; maybe four base pairs are involved or maybe there is some sort of ambiguity as to which of the two possible triplets in the run of four the tRNA responds to. Bossi and Roth however, now describe a new suppressor, SufJ, which responds to sequences of four bases: ACCU, ACCC and ACCA (the sequence ACCG has not been tested). In other words it recognizes the triplet ACC but pulls the fourth base through the reading frame. This seems to be a sort of three out of four reading mechanism reminiscent of Lagerkvist's idea.

Simple explanation to account for these puzzling phenomena are not in sight but one thing seems certain: our once clear picture of the mechanism underlying the translation of the genetic message is developing a plethora of unbecoming flaws.

## Coding complications

from a Correspondent

READERS will recollect that the celebrated wobble hypothesis of Crick defines the nature of the interaction between the codons of messenger RNA and the corresponding anticodons of tRNA. In short, the codons are read one at a time by tRNA molecules. These form three base pairs between the bases in their anticodon and those in the codon that it responds to. The first two base pairs formed by the codon are standard Watson-Crick A-U or G-C pairs but in the third position G-U pairs are allowed, as are A-I, G-I and U-I where the I residue is in the anticodon.

Although there is considerable evidence that this is the normal mode of codon-anticodon interaction in both eukaryotic and prokaryotic organisms, evidence has been accumulating that things are not always as simple as this. Thus in mitochondria, it would appear that a single base in the third position of the anticodon can 'read' each of the four bases which might occur in the third position of a codon, or to put the matter another way, only the first two bases of the codon are relevant. This state of affairs resembles the 'two out of three' mechanism proposed by Lagerkvist<sup>1</sup>. More evidence for such anomalies now appear in two papers in *Nature* and in two in *Cell*.

Munz *et al.* (see this issue of *Nature*, p. 187) have studied serine specific tRNA in *Schizosaccharomyces pombe*. This organism utilizes two isoaccepting tRNA<sup>Ser</sup> with anticodons IGA and U\*GA (U\* is a modified uridine) respectively. The one with IGA should be able to respond to the codons UCU, UCC and UCA and the other only to UCA. By means of some clever genetic manipulations Munz *et al.* were able to show, in essence, that mutants in which the U\*GA species is not synthesized are not viable. If the possibility that the missing tRNA had some other essential role to play is excluded, this would seem to imply that UCA codons were not being

properly recognized. The anomaly appears when it is noted that the other serine specific isoacceptor with IGA for its anticodon should have been able to recognize this codon according to the rules of wobble. Apparently it does not. The significance of this *in vivo* result is increased when it is realized that the bulk of the evidence for A-I interactions is derived from *in vitro* experiments.

Meanwhile, Diamond, Dudock and Hatfield<sup>2</sup> have isolated and sequenced an unusual tRNA species from bovine liver. This accepts serine but has CmCA for its anticodon, an anticodon normally expected to recognize the tryptophan codon UGG. The anomaly appeared when it was found from *in vitro* binding experiments that the tRNA responded to UGA (which is normally the opal stop signal) but not to UGG or any of the serine codons. It was further shown that the tRNA could act as a suppressor of termination as the first result might suggest. In other words the Cm in the third position had 'read' A rather than G as demanded by the wobble hypothesis. This result for a eukaryote had been anticipated in the work of Hirsch<sup>3</sup> who described a tRNA<sup>Trp</sup> in *E. coli* which had an anticodon CCA but which responded to UGA codons. Both the eukaryotic and the prokaryotic tRNA molecules also had anomalous features in their sequences in the vicinity of their DiHU stems and the possibility remains that this feature is somehow related to their anomalous codon reading properties.

Another curious anomaly has been discussed by Bienz and Kubli (see this issue of *Nature* p 188). *Drosophila melanogaster*

1. Lagerkvist *Proc. natn. Acad. Sci. U.S.A.* **75**, 1759 (1978).
2. Diamond, Dudock & Hatfield *Cell* **25**, 497 (1981).
3. Hirsch *J. molec. Biol.* **58**, 439 (1971).
4. Bossi & Roth *Cell* **25**, 489 (1981).



# Eight new Nobel laureates

## A spectrum of achievements

from David D. Burgess

One half of the Nobel Prize in Physics was awarded jointly to *Nicolaas Bloembergen*, Harvard University and *Arthur L. Schawlow*, Stanford University, for their contribution to the development of laser spectroscopy, and the other half to *Professor Kai M. Siegbahn*, Uppsala University for his contribution to the development of high-resolution electron spectroscopy.

SPECTROSCOPY, the study of all aspects of the interaction of electromagnetic radiation with matter in all its forms, not necessarily simply atomic or molecular, (undergraduate memories of thousands of atomic energy levels notwithstanding!), remains by its very nature one of the most powerful tools in large areas at the frontiers of both physics and chemistry. What is more interesting is that the prize has been given to this year's three Nobel laureates not for a specific scientific application, but for a more general and extended series of developments in technique. For obvious physical reasons the first demonstrations of methods of the type pioneered by the three Nobel winners have been in relatively 'pure' areas of atomic and molecular physics. However, the likely impact is much wider both scientifically and with important uses already developed in some technological and industrial spheres.

Schawlow and Bloembergen share half the prize for developments in laser spectroscopy. Some spectroscopic applications of lasers, of course, are simply extensions in kind of conventional techniques. Obvious increases in spectral resolution (from the very narrow bandwidths available), in signal (from high input intensities) or in time-resolution (from short pulse length) have been widely exploited. However, what Schawlow himself termed "The Laser Revolution in Spectroscopy" goes much deeper, stemming from two separate features which, in the visible and UV, occur only when laser sources are used. One is the ability to reach intensities at which the interaction of an atom or molecule with the electromagnetic field dominates that of all other interactions with its environment, whether radiative or collisional. The second is that the interaction of the atom or molecule with the electromagnetic field itself may become nonlinear.

Schawlow's achievements primarily relate to the first point. The power of spectroscopy becomes greatly enhanced if specific atoms or molecules in an ensemble can be 'tagged' in order to distinguish them from their surroundings, for example, by

selectively exciting only specific chemical or isotopic species, specific velocity sub-groups, specific bound states, and so on. Almost the only way to do this is by interaction with laser radiation. A trivial calculation shows that at optical frequencies, thermal light sources, however bright and well-focused, cannot in general provide irradiances sufficient to cause significant selective excitation in the face of the always competing spontaneous radiative decay of the selected atomic or molecular state. Other excitation methods, for example, with pulsed electron beams, are not usually state selective. However, laser radiation provides both the intensity and the spectral resolution to change any selected state population in a sample at will, almost regardless of competing processes, and therefore to 'tag' chosen atoms.

When widely tunable lasers first became available in the late 1960s, Schawlow (a progenitor of the laser itself in his earlier work with Townes) left the then fashionable topic of laser development largely to others and with his group concentrated on a series of spectroscopic applications, often of a devastating simplicity and elegance. Prime amongst these was the development of 'Doppler-free' spectroscopy, by saturation spectroscopy, by 2-photon excitation, and by polarization spectroscopy, in which limitations on resolution always set previously by the thermal motions of atoms and molecules in a sample were removed either by selective excitation of particular velocity sub-groups, or in 2-photon spectroscopy by the actual cancellation of the Doppler shift regardless of the individual absorber's velocity.

The impact of the consequent vastly-increased resolution on atomic and molecular physics is obvious, with a re-measurement of the Rydberg to increased precision by the Stanford group, a greatly increased ease of observing, for example, isotope shifts or high quantum states now exploited by many groups worldwide, and proposals even for looking at aspects of atomic physics related to current Unified Field Theories. Schawlow and his collaborators also demonstrated other possibilities of the immense sensitivity of laser spectroscopy, for example the capability of detecting just one atom of a given species against a background of other

species of almost arbitrary density, at one step not only pushing back the boundaries of chemical trace analysis by several orders of magnitude but indeed extending them to their ultimate physical limit. More recently, Schawlow has developed spectroscopic methods simplifying the analysis of molecular structure by selectively laser-tagging specific states.

Many of these spectroscopic developments have applications far outside pure atomic and molecular physics. The most obvious case is Doppler-free spectroscopy which is very intimately related with some schemes for obtaining efficient laser separation of fissile isotopes. However, many of the applications of the vast increase in spectroscopic sensitivity remain to be explored. (A single but rather nice example, given in a review by Letokhov at the recent Heidelberg European Atomic Physics meeting, is that the enormous sensitivity of laser spectroscopy for measuring relative isotopic abundance may now render all other radiocarbon and related dating methods quite obsolete.)

Bloembergen's work includes both theory and experiment in areas very similar to those described above, but perhaps best known is his contribution to the analysis of the possible nonlinear interactions of an atom or molecule with the electromagnetic field itself when the field intensity is high. The number of such nonlinear processes is vast. As well as fundamental interest many already have technological applications, whether to parametric losses in optical communication systems, spectroscopic analysis of jet engine exhausts via CARS (coherent anti-Stokes Raman Spectroscopy), atmospheric self-focusing of high-intensity laser beams due to nonlinear refractive indices, or recent developments of 'phase-conjugate' reflectors opening the possibility of optical systems in which aberrations and defects, however severe, are automatically self-cancelling. Important spectroscopic applications result from the availability of frequency up- or down-converted coherent sources in difficult spectral regions such as the vacuum ultraviolet and also from the more direct use of related '4-wave mixing' processes such as CARS and its derivatives as immensely sensitive spectroscopic tools.

The theoretical prescription provided by Bloembergen and his group, notably in a crucial paper in 1962, provided a suitable framework for distinguishing between and analysing the many possible competing nonlinear effects, particularly processes leading to frequency up or down

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conversion, laying an immensely timely foundation for the type of applications already mentioned. Lately, Bloembergen has also become interested in a further technological possibility of selective laser spectroscopy of molecules, that of laser-induced chemistry, or laser catalysis, in which the direction and speed of a chemical reaction may be controlled by selective excitation of specific states in the reactants, or by selective destruction of the reactant products.

Laser spectroscopy has taken a large share of the fashionable limelight in recent years, but the award of half of the prize to Professor Kai Siegbahn is a reminder of equal recent progress in areas where lasers are not yet available, particularly the VUV and X-ray regions. Siegbahn's achievements relate to specific uses of photoelectron spectroscopy, the accurate measurement of the energies of electrons ejected by atoms or molecules irradiated with VUV or X-ray wavelengths. Siegbahn's contribution has been ESCA, Electron Spectroscopy for Chemical Analysis. In the complex spectra of most molecules, the structure of the individual atomic valence states is so perturbed by the bonding process that even in simple cases the visible or ultraviolet spectrum is not usually an *a priori* guide to the atomic constituents. Siegbahn realized that this could be overcome at X-ray wavelengths, the inner atomic electrons being largely shielded from the molecular environment, and the excitation energies therefore remaining characteristic of the atomic species involved. However, with laser techniques yet unavailable at X-ray wavelengths, orthodox emission or absorption spectroscopy does not provide a full basis for such an analytic method, emission requiring temperatures frequently sufficient to destroy the molecule of interest, and absorption work often needing excessively large samples. Siegbahn, therefore, used the alternative technique of energy analysing electrons ejected from the inner shells of atoms in molecules irradiated with specific X-ray wavelengths, a technique applicable to relatively small samples. The spectra so obtained additionally allow the investigation of the particular bonding environment of each atomic species, as small 'chemical shift' of the inner-shell binding energies by outer bonding electrons remains sufficient for aspects of the bond to be characterized without loss of the atomic identity of the spectra needed for direct chemical analysis.

In the past fifteen years spectroscopy has ceased to be simply a powerful but established technique; in some ways changing technically more than at any time since Newton. A huge challenge remains in both pure and applied science in the yet untapped applications of the type of techniques developed by the three Nobel winners in what now almost constitutes a completely new subject. □

## Hemispheric modes of consciousness in the human brain

from Colwyn Trevarthen

One half of the Nobel Prize in Physiology or Medicine was awarded to *Roger W. Sperry*, California Institute of Technology, for his discoveries concerning the functional specialization of the cerebral hemispheres.

ROGER SPERRY, a leader among students of the functional design of the brain for forty years, might well have received the Nobel Prize for experiments he did in the 40's on the self-sorting of nerve pathways regrowing after surgical rearrangements in fish and amphibia. Many neurobiologists still see that as his greatest work.

The split-brain studies for which he received the prize last month led to discoveries about the human brain which are, however, of far greater importance for psychology. They showed that each human cerebral hemisphere, when disconnected from the other by surgery, could formulate consciousness, feelings and intentions on its own and that left and right sides had different mental abilities.

The experiments with human subjects came directly out of micro-surgical research at the University of Chicago on the transfer of learning from one side of the brain to the other in fish. When Sperry and Ronald Myers applied this approach to cats in the 1950's, they achieved the first convincing surgical duplication of the brain systems serving memory formation and awareness in one animal. Their brilliant, microscopically controlled operations sliced through the cross-over of visual input pathways under the brain, and the corpus callosum between the cerebral hemispheres.

Sperry's studies of split-brain cats and monkeys continued at the Californian Institute of Technology after he became Hixon Professor of Psychobiology there in 1954.

Then, in the 1960's, the elegant behavioural tests he had already devised for animals showed Sperry the way to effective study of human commissurotomy patients. This gave the breakthrough to clearer understanding of specifically human states of mind.

A Los Angeles neurosurgeon, Joseph Bogen, observing the rapid recovery of monkeys after Sperry's split-brain surgery, recalled that the corpus callosum of a human being could be transected safely in selected patients with uncontrollable epilepsy, to interrupt the seizures that were devastating these peoples' lives. A small

group of patients were operated upon by Philip Vogel and Bogen during the 60's producing a successful alleviation of their epilepsy and a marked improvement in their health. Sperry worked with Bogen and a number of graduate students, of whom Michael Gazzaniga was the first, in the discovery of a most extraordinary division of human consciousness in these persons: a duplicate state of mind largely hidden when the patients were in free mastery of their experience and actions. Sperry led a systematic exploration of the full scope of this effect and so clarified a uniquely human division of mental tasks between the left and right halves of the brain.

That parts of the left hemisphere are dominant in the control of speaking and writing and for understanding language was discovered in the last century. Observation of the psychological effects of wounds in different parts of the cortex produced evidence for each and all of the hemisphere differences that Sperry disclosed in his investigation of commissurotomy patients. But the history of this quest did not allow a coherent overview. There had never been a brain chart like Sperry's which summarized the basic division of functions, giving equal attention to both hemispheres. Even though a group of psychologists had been saying since the 40's that the right hemisphere could be superior to the left in certain non-verbal visuo-spatial kinds of detection and reasoning, their point was not well-taken by the majority of neurologists who were inclined to identify human intelligence with verbal intelligence.

The tests devised by Sperry's group showed that an isolated left hemisphere had clear superiority for functions linking a sequential pattern of logic with the production of speech and writing. They also showed that right hemisphere consciousness was not a weak copy of the left. It could perform some tasks better than the left even though it could not use elaborate verbal imagery or rational thinking of the propositional variety. Thus, the tests clarified our intuitive non-verbal insight and concrete awareness and drew attention to the work the brain must

Accounts by R.W. Sperry of his work are found in *Cerebral Organization and Behaviour*, *Science* 133, 1749 (1961); *Mental unity following surgical disconnection of the cerebral hemispheres*, *Harvey Lectures* 62, 293 (1967); *Mind-Brain Interaction: Mentalism, Yes: Dualism, No*, *Neuroscience* 5, 195 (1980); *Changing Priorities*, *Annual Review of Neuroscience* 4, 1 (1981).

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do to directly perceive and understand persons and things in the humanly ordered world about us. The right hemisphere was shown, for example, to be strongly preferred for feeling strange shapes in the hand, for copying drawings and for recognizing faces.

Sperry and his students, notably Jerry Levy, Robert Nebes, Harold Gordon, Eran and Dahlia Zaidel and the writer, adapted psychological tests to show how a split-brain person could be conscious with one half of the cerebral cortex at a time, or how he or she might choose to distribute a given cognitive task between the two disconnected halves. In all this work, which has produced many renowned scientific papers, Sperry acted as navigator and critic, employing his remarkable literary skills to draw the pieces of the puzzle into a bold harmony of primary principles.

Sperry has written important papers on the philosophical implications of split-brain research, regarding the casual potency of consciousness as a spatio-temporal brain process that creates reality, goals, values, knowledge and skills in brain organs with a definite inherited anatomical design. Cerebral anatomists

are now actively seeking inborn differences in the organization of cortical tissue in the two hemispheres. Psychology is encouraged to look inside the mind to understand internal and preordained standards of 'will', 'desire', 'imagination' and 'understanding' that condition how experience will affect our actions.

The studies of split-brain patients have opened the way, also, to a developmental approach or to an embryology of the mind. Human cognitive systems grow over many decades. Interaction of the hemispheres while they develop in the fetus and through childhood results in a segregation of major functional systems in a partnership or federation. Power sharing is thereby achieved with a much higher efficiency in the integration of an individual's purposes and adaptive actions. This process takes a different course in right and left handers, in males and females, educated and uneducated, the blind and the deaf. There takes place in each of us a progressive division of brain work and generation of a unique bihemisphere condition. Genetically based hemispheric specialisations enter into regulation of the individual's place in his family, his society, his culture and in the history of his community. □

been known that the contralateral eye sends fibres to laminae 1, 4 and 6 in the nucleus which relays information to the visual cortex, with laminae 2,3 and 5 supplied by the ipsilateral eye. Hubel and Wiesel made electrolytic lesions limited to a single lamina in the relay nucleus and found degenerating nerve endings in a series of regularly spaced bands about 400  $\mu$  wide and separated by 400  $\mu$  throughout the visual cortex. If their lesion in the relay nucleus encroached on an adjacent lamina, the cortical degeneration became continuous.

The existence of right and left eye ocular dominance columns was fully confirmed by other methods, and they have been found in cats, galago and the macaque, but not in the mouse, the tree shrew or the squirrel monkey. The basic unit seems to be an about one millimetre square 'hypercolumn', containing left and right ocular dominance columns crossed approximately at right angles by about ten orientation columns.

It was previously known that retinal fibres project on the visual cortex in an orderly fashion, so that in man the fovea, the point of most distinct vision, is represented at the occipital pole and the extreme periphery at the front end of the calcarine fissure, and it was known from Gordon Holmes's work that the fovea had a disproportionately large area of representation in the cortex. It became clear that a hypercolumn of one square millimetre in the foveal area would deal with a solid angle of 10 minutes of arc, whereas a similar hypercolumn in the far periphery would cover about 10 degrees. There is a continuous representation of the visual field along layer IV in each ocular dominance column, half overlapped by the representation in the adjacent column for the other eye. In this way topographical representation, provenance from each eye and orientation are all represented in a two dimensional sheet of cortex.

Hubel and Wiesel have also established that ocular dominance and orientation preference is present at birth in cats and monkeys without visual experience. New-born animals differ from adults in having a less sharp tuning of orientation preferences and less binocular interaction. These differences seem to be due to lack of visual experience rather than immaturity as animals whose eyes are kept closed for some weeks from birth show little improvement on the state at birth. The system is still plastic, however, since closure of one eye soon after birth results in the take-over of most of the cells of layer IV by afferent fibres from the open eye so that after some weeks the deprived eye drives very few cells in the cortex. This plasticity lasts for about three months in kittens, somewhat longer in monkeys and up to one to two years in man. Closure of one eye after this 'critical period' has no permanent effect and reopening and retraining must take place during the critical period if there

## Visual machinery of the brain

from David Whitteridge

One half of the Nobel Prize in Physiology and Medicine was awarded jointly to *David H. Hubel* and *Torsten N. Wiesel*, both of the Harvard Medical School, for their discoveries concerning information processing in the visual system.

THE work of Hubel and Wiesel over the last twenty years has established two main points: first, that the visual cortex has a remarkably ordered fine structure, composed, in the monkey, of about a thousand units of identical structure, each of which contains mechanisms for the analysis of all the data from corresponding parts of the visual fields and, second, that these orderly units are very largely established by genetic mechanisms during prenatal development and can be modified by visual experience only during the first few months of life.

Their first major discovery was that cortical visual cells are excited by bars, light or dark, or by single edges, and that each cell responds optimally to a particular orientation of the stimulus. This was previously unsuspected, as ganglion cells of the retina and the cells of the lateral

geniculate (relay) nucleus were known to have round fields, usually concentrically arranged so that an excitatory centre would be surrounded by an inhibitory surround and vice versa.

To analyse all possible patterns it would be necessary to have sets of cells sensitive to a complete range of orientations. In the cortex such an organization has been found. Cells of one orientation occupy a "column" about 50  $\mu$  wide, extending from the surface to the white matter, with columns of approximately ten degrees different orientation, displaced clockwise or anticlockwise, on each side. A horizontal penetration through the cortex encounters a regular series of such orientation columns occasionally interrupted by reversals. Much later, Hubel and Wiesel were able to display the full extent of orientation columns by injecting radioactive deoxyglucose, and exposing the animal to light bars at a single orientation. A series of regularly spaced thin curved columns were found to be stained.

They established a second independent set of 'ocular dominance' columns each of which received fibres from one eye only and contained cells which were driven exclusively (in cortical layer IV) or predominantly by that eye. It had long

is to be any recovery. The mechanism by which active fibres can apparently displace inactive fibres is obviously of great importance and as yet not understood.

This knowledge of the organisation of

the primary visual cortex is being used to understand the activities of other visual areas and is forcing reconsideration of present ideas on the *modus operandi* of all other cortical areas. □

since their reactions are largely those of the delocalised pi-electron system built from atomic 2p atomic orbitals.

Hoffmann's first major contribution to theoretical chemistry was to extend the formalism of the pi-electron Hückel theory to include sigma electrons. Thus molecules of the aliphatic type such as ethane could be considered as well as aromatics such as benzene. This simple semi-empirical molecular orbital technique is still widely used since it can treat very large molecular systems without demanding anti-social amounts of computer time.

Experience gained from extended Hückel calculations led to the now famous Woodward-Hoffmann rules which Fukui showed could be explained by frontier orbital theory, (although there are other explanations including symmetry arguments). The idea of Hoffmann and the late Nobel laureate R.B. Woodward explains why, for example, maleic anhydride reacts easily with butadiene but not at all easily with ethylene. In the former case two new carbon-carbon bonds are made simultaneously and the electrons which are involved complete a circuit. Concerted and cyclic reactions of this type are called pericyclic and this example is a cycloaddition. Woodward and Hoffmann's rules start with a consideration of the molecular orbitals of the reactants and match up the electron densities in occupied and unoccupied molecular orbitals. A correlation diagram permits a simple decision to be made as to whether a particular reaction is allowed or not.

Vast numbers of applications of the Woodward-Hoffmann rules are to be found in recent literature and perhaps the best testament to the power and range of applicability of these rules is to quote in its entirety the first paragraph of chapter 12 from the eponymous pair's book *Conservation of Orbital Symmetry*; Chapter 12, Violations "There are none!" □

## Rules for chemical reactions

from Graham Richards

One half of the Nobel Prize in Chemistry was awarded to *Kenichi Fukui*, Kyoto University and the other half to *Ronald Hoffmann*, Cornell University, for their theories concerning the course of chemical reactions.

THEORETICAL CHEMISTS often seem to work at one of two extreme ends of the same problem. Either they do highly accurate calculations on small molecules or crude, even qualitative, investigations of larger molecules which are closer to the molecules synthesized and used at the laboratory bench. Both Fukui and Hoffmann belong to the latter school. Their work has added an excitement to broad areas of organic chemistry. The culmination of their research in the form of the Woodward-Hoffmann rules represents what is perhaps the most significant single step in organic chemistry in the post-war period.

The chemistry of a molecule is determined by the distribution of its electrons and by how tightly these electrons are held at particular locations in the molecule. Fukui's method of frontier molecular orbitals concentrates on the electrons which are the most loosely bound. If a reactant is involved in donating electrons to form bonds then the electrons

involved are most likely to be those most easily removed. Conversely, if a molecule reacts by virtue of acceptance of electrons into a previously unoccupied molecular orbital then the bond will be created in the region where the extra charge is most readily accepted. Frontier molecular orbital theory then looks quantitatively at electron density distributions in the occupied molecular orbital which is highest in energy terms and at the lowest unoccupied molecular orbital. The new acronyms HOMO and LUMO abound in current literature on organic and medicinal chemistry.

Frontier orbital theory is based on the perturbation treatment of molecular orbital theory introduced by Coulson and Longuet-Higgins. It deduces conclusions about chemical reactions by looking at the interaction of the reactants, assuming that the position of the transition state on the potential energy surface can be deduced from the initial slope along the reaction coordinate; the smallest slope is identified with the lowest energy transition state and consequently the fastest reaction or preponderance of products. Fukui's ideas were developed and exploited in the early days when theoreticians were not blessed with large computers and in consequence restricted to pi-electron systems and the simple Hückel molecular orbital method. This limitation nonetheless allows consideration of the reactivities of all the aromatic molecules of organic chemistry

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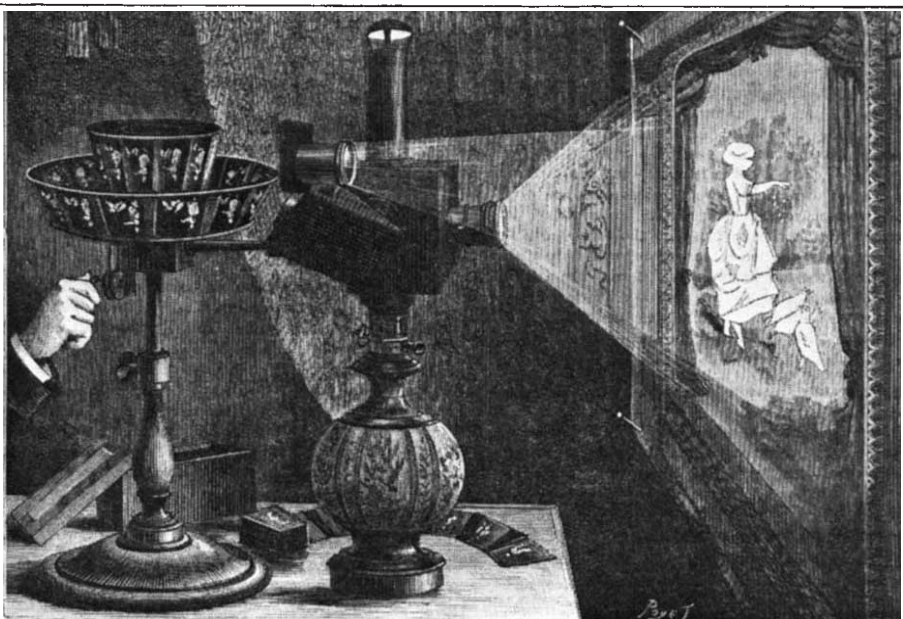


99 Years ago

### THE PROJECTION PRAXINOSCOPE

M. Gaston Tissandier describes in *La Nature* an ingenious adaptation of the praxinoscope by means of which the images are projected on a screen, and are visible to a large assembly. M. Reynaud, the inventor obtains at once the projection of the scene or background — by the object-glass which is seen at the side of the lantern — and of the subject, by another object-glass which is shown in front of and a little above the same lantern. In making the two parts of the apparatus converge slightly, the animated subject is brought into the middle of the background, where it then appears to gambol. A hand-lever on the foot of the instrument allows a moderate and regular rotation to be communicated.

From *Nature* 27, 61, November 16, 1882.





## REVIEW ARTICLE

# A new era in mammalian gene mapping: somatic cell genetics and recombinant DNA methodologies

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*Mammalian gene mapping techniques are now sufficiently advanced to contribute significantly to prenatal diagnosis and to human molecular genetics. Restriction fragment mapping can be used to place polymorphic genetic markers at random sites within the genome, and these sites used to assign genes responsible for disease conditions to a chromosomal region. Somatic cell genetic techniques can then be applied to saturate that region with additional restriction fragment markers, some of which will be closely linked to the disease gene. Closely linked restriction fragment markers, especially flanking pairs of markers, can act as predictors for the transmission of defective genes to offspring. A series of tightly linked flanking restriction markers might in addition contribute to the eventual isolation and cloning of the disease gene itself.*

SOMATIC cell genetics provides a means of analysing the complex genetic organization and regulation of higher eukaryotes<sup>1,2</sup>. Although it has contributed significantly to our knowledge of mutagenesis<sup>3</sup>, genetic complementation<sup>4</sup> and gene regulation<sup>5</sup>, its major success has been in gene mapping, especially in man<sup>6,7</sup>. Two approaches to the analysis of the human genome have been remarkably successful. Recombinant DNA techniques have allowed several genes and their flanking DNA regions to be sequenced, and using such techniques, functional regions, such as promoters for RNA synthesis, have been identified and mutations and deletions precisely defined. At the level of the whole chromosome somatic cell genetic techniques have been used to map hundreds of human genes directly, and hundreds more have been mapped on the basis of known linkages to the first group. Until recently analysis of the intermediate level of human genetic analysis has been much less successful. Now, however, a variety of approaches make this range accessible as well. This review focuses on these approaches and on the way in which their concerted application will yield both a better understanding of eukaryotic genome structure, and information useful in clinical diagnosis.

## Gene transfer between mammalian cells

Gene mapping by somatic cell genetics depends primarily on parasexual events which facilitate the transfer of genetic material between cells (gene transfer) and the loss of transferred material from hybrid cells (segregation). In discussing gene transfer systems, it is convenient to distinguish between the *donor* cell which serves as the source of transferred genetic materials and the *recipient* cell into which donor genetic material is delivered. The resulting heterogenote is referred to simply as the *hybrid* cell. There are four ways of delivering genes into recipient cells:

(1) Cell hybridization: Fusion of two genetically distinct parental cells (for example, man × mouse) yields cell hybrids which contain initially complete parental chromosome sets. In such interspecific hybrids, one chromosome set is usually partially lost (segregation), and for convenience this set is termed the donor set. Hybridization thereby provides a means of transferring large numbers of donor chromosomes into a recipient cell. Hybrid clones can be established which retain a defined set of donor chromosomes.

(2) Microcell-mediated gene transfer: Donor cells can be manipulated so that their chromosomes become isolated in microcells<sup>8</sup>, which may possess only one or a few donor chromosomes. Such cells can be hybridized to recipient cells, and thereby serve as convenient vectors for the transfer of one or a few intact donor chromosomes into recipient cells. An advantage of the microcell approach is experimental control over chromosome segregation.

(3) Chromosome-mediated gene transfer: Cell-free preparations of donor chromosomes can be used to transform recipient cells<sup>9-10</sup>. In this system, whole chromosomes are taken up by endocytosis and then degraded into subchromosomal fragments. Fragments large enough to be detected by light microscopy as well as submicroscopic fragments have been described<sup>11</sup>. The lower limits of fragment size have not yet been determined.

(4) DNA-mediated gene transfer: Eukaryotic cells can be transformed genetically by purified DNA<sup>12</sup>. Two primary means of transfer exist: endocytotic uptake of calcium phosphate precipitated DNA, and direct injection of purified DNA into the nucleus by microcapillary pipettes. Transformation frequencies obtained by microinjection can be as high as 20% (ref. 13), whereas transformation by endocytosis of precipitated DNA (or the endocytotic uptake of chromosomes) is generally four to five orders of magnitude less efficient. In DNA-mediated transfer, the size of the donor fragment initially taken up by the recipient cell is invariably small, usually less than 50 kilobases (kb).

Thus it can be seen that through the choice of the particular gene transfer system used one can control within broad limits the amount and size of donor material transferred into a recipient cell. As we shall see below, this capability provides a means of regulating the resolution of gene mapping analysis.

## Gene mapping by somatic cell genetics

The assignment of genes to chromosomes and regions of chromosomes in somatic cell genetic systems is accomplished by correlating a particular donor gene or its product with a specific donor chromosome or a subchromosomal fragment<sup>14</sup>. More than 200 human genes have been assigned to particular chromosomal locations in the human genome by this method, bringing the total number of mapped genes in man to more than 400. On the average, four human genes are mapped per month

by the somatic cell approach, and as we shall see, this accession rate can be expected to increase dramatically in the next few years.

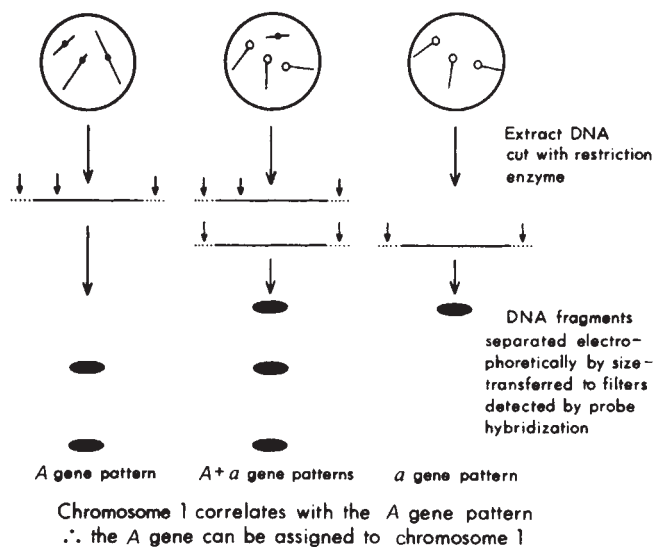
Until recently, the method of choice in gene assignment was the correlation of a particular gene product (such as an enzyme, structural protein or surface antigen) with an identifiable donor chromosome or fragment. This method has severe limitations, because it depends on the expression of the donor gene and thus precludes the mapping of genes which are expressed only in certain differentiated cells or of nucleotide sequences which have no coding functions. These drawbacks can be overcome by using nucleic acid hybridization techniques to detect donor genes directly. We first showed the feasibility of this approach in the assignment of the human  $\alpha$ - and  $\beta$ -globin loci using solution hybridization ( $C_{ot}$  analysis) of  $\alpha$ - and  $\beta$ -globin cDNAs with DNA preparations from hybrid clones containing different human chromosomes. These studies showed that the  $\alpha$ -globin complex mapped<sup>15</sup> to human chromosome 16, and the  $\beta$ -globin complex mapped<sup>16</sup> to human chromosome 11. The success of this method depends on the specificity of hybridization of the donor gene probe with the homologous donor human genomic sequence, and so appreciable cross-reaction with recipient cell DNA sequences will limit the use of solution hybridization as a gene mapping technique. This represents a serious restriction, because in many instances cross-hybridization of structural gene probes can be expected to occur, as we found to be the case when we attempted to extend the solution hybridization procedure to the assignment of the murine immunoglobulin genes. In this system, there was extensive cross-hybridization between cloned recombinant DNA probes of specific mouse immunoglobulin nucleotide sequences and the recipient (in this case, Chinese hamster) genomic sequences. This problem was solved by making use of donor and recipient differences in restriction endonuclease sites within or around the gene of interest and using the Southern blotting procedure to reveal these differences (Fig. 1). The differences in the location of restriction sites are diagnostic for the donor and recipient forms of a particular immunoglobulin gene. Using this approach, it was possible to assign the mouse  $\kappa$ ,  $\lambda$  and heavy chain complexes to chromosomes 6<sup>17</sup>, 16<sup>18</sup> and 12<sup>19</sup> respectively. The restriction fragment mapping procedure has the advantage that it is independent of hybridization specificity, depending only on the restriction fragment length differences which occur frequently between species. This approach makes possible the mapping of any unique or low copy number nucleotide sequence, and within

a relatively short period, an impressive number of gene complexes have already been assigned in human and murine genomes using this procedure (Table 1). As the number of cloned DNA probes to specific genes is expanding rapidly, we can expect the mapping of such genes to increase commensurately.

Chromosome cloning techniques (that is, the isolation of single donor chromosomes in a host recipient cell) can also be used to assign a gene to small subregions on a particular chromosome and to establish the order of different genes along the length of the chromosome<sup>20</sup>. This can be accomplished by using donor cells which carry reciprocal translocations involving a particular chromosome. Such translocations occur at a relatively high frequency in human families where they are detected as a consequence of high abortion rates or congenital defects. A concerted effort has been made to collect cells from translocation carriers<sup>21</sup>; more than 300 such translocations have been stored, and for each the translocation breakpoints have been described in terms of chromosome banding patterns. If a gene has been assigned to a particular chromosome, then a series of translocations involving different sites along the chromosome can be used to delimit the position of the gene. This position has been termed the 'smallest region of overlap' (SRO)<sup>22</sup>. Translocations or deletions which arise spontaneously in the hybrid cells can also be used for this purpose. More than 100 human genes have been assigned to subchromosomal regions by these methods. Such regional mapping procedures lend themselves readily to restriction fragment mapping; for example, the human  $\beta$ -globin locus was assigned to the chromosomal subregion 11p125-p128 in this way<sup>23</sup>.

Human reciprocal translocations can also be used in conjunction with chromosome sorting techniques to fix the subchromosomal localization of genes. It has been shown that isolated mammalian mitotic chromosomes can be sorted into discrete size groups by fluorescence particle sorting<sup>24</sup>. Nearly every human chromosome can be resolved as a unique size class by this technique. DNA can be isolated from the sorted chromosomes and then hybridized with cloned probes in order to establish gene assignments. Translocations are particularly useful in this mapping procedure because they alter the size of a chromosome in a predictable way, causing a change in the sorting pattern, and thus enabling a suspected linkage relationship to be verified<sup>25</sup>. Drawbacks of the chromosome sorting approach involve the relatively long sorting times required for recovery of sufficiently large samples and the high cost of the procedure. Despite these limitations the technique can be expected to add another dimension to gene mapping methodologies.

Considering all the regional mapping procedures, probably the greatest potential lies with *in situ* hybridization<sup>26</sup>. This technique is used routinely to locate single copy genes in *Drosophila* polytene chromosomes, where they are amplified about 1,000 times. The older *in situ* hybridization procedures could reliably detect single copy genes amplified as little as 100-fold—but not less. Recently new procedures have been introduced which significantly increase the sensitivity and resolution of the method so that a single copy gene amplified 10–20-fold can be detected<sup>27</sup>. It may also be possible to detect unamplified unit genes using additional refinements; in fact, several investigators have claimed success in mapping single copy genes by *in situ* hybridization<sup>28–31</sup>. Recent innovations include: the use of <sup>125</sup>I-labelled nucleotide, introduced into DNA probes by nick translation, which produces radioactive probes of high specific activity (up to 10<sup>9</sup> d.p.m. per  $\mu$ g DNA)<sup>27</sup>; the use of dextran sulphate in the hybridization reaction mixture to increase hybridization efficiency<sup>32</sup>; single-stranded probes which do not hybridize to themselves, thus providing an additional increased efficiency of approximately five- to seven-fold<sup>33</sup>. More recently, it has been shown that biotin derivatives of cytidine can be produced (D. Ward, personal communication), which can be efficiently incorporated into probes by nick translation. DNA containing biotinized nucleotides can



**Fig. 1** Restriction fragment mapping. The restriction pattern of the donor cell gene (A) differs from that of the recipient cell gene (a). The probable region of the genes is depicted by solid lines. Diagnostic fragments of different molecular weight are generated from the two genes by endonuclease digestion. The signature of the donor gene can be correlated with a particular donor chromosome.



**Table 1** Recent progress in restriction fragment mapping

| Human               |                                       |        | Mouse               |                                              |      |
|---------------------|---------------------------------------|--------|---------------------|----------------------------------------------|------|
| Chromosome position | Gene                                  | Ref.   | Chromosome position | Gene                                         | Ref. |
| 2p                  | Proopiomelanocortin                   | 54     | 3                   | r-protein L30                                | 62   |
| 6                   | Prolactin                             | 55     | 5                   | r-protein S16                                | 62   |
| 9                   | $\alpha$ - and $\beta_1$ -interferons | 56     | 5                   | J protein                                    | 63   |
| 11                  | Insulin                               | 57     | 5                   | Casein complex                               | 67   |
| 11p125-p128         | $\beta$ -Globin complex               | 23     | 5                   | $\alpha$ -Fetoprotein                        | 65   |
| 11p11-p13           | Four anonymous restriction            | 50     | 6                   | r-protein L19                                | 62   |
| 11p128-p11          | fragments                             |        |                     |                                              |      |
| 11p13-p128          |                                       |        |                     |                                              |      |
| 11p13-pter          |                                       |        |                     |                                              |      |
| 17q21-qter          | Growth hormone complex                | 58, 59 | 6                   | $\kappa$ Light chain immunoglobulin complex  | 17   |
| X                   | Unknown restriction fragments         | 61     | 7                   | r-protein L18                                | 62   |
|                     |                                       |        | 11                  | $\alpha$ -Globin complex                     | 66   |
|                     |                                       |        | 12                  | Heavy chain immunoglobulin complex           | 19   |
|                     |                                       |        | 12                  | r-protein L18                                | 62   |
|                     |                                       |        | 12                  | r-protein L30                                | 62   |
|                     |                                       |        | 16                  | $\lambda$ Light chain immunoglobulin complex | 18   |
|                     |                                       |        | 17                  | $\alpha$ -Globin pseudogene $\psi 3$         | 66   |
|                     |                                       |        | 18                  | $\alpha$ -Globin pseudogene $\psi 4$         | 66   |

be used as antigens to produce specific polyclonal or monoclonal anti-DNA antibodies. Such antibodies can be fluorescently, isotopically or enzymatically labelled, and then used as a second label against the polynucleotide probe. Labelled antibodies directed against the anti-DNA antibodies can also be produced and used to increase signal intensity.

Theoretically, combinations of the above techniques should permit the regional localization of unit genes. The *in situ* method is all the more promising when one considers that many gene loci exist as complexes containing clusters of related genes. If mixtures of the relevant probes were used, one would expect a corresponding increase in sensitivity. It may also be possible to use the *in situ* technique in combination with restriction fragment mapping of chromosomes using cell hybrids. For example, a cloned probe could be used initially to map a gene to a particular chromosome using the restriction fragment mapping technique, and then the same probe could be used *in situ* to localize the gene to a specific region on the chromosome. The advantage of the sequential procedure is that only one chromosome need be considered in the *in situ* analysis, thus reducing the possibility of error due to nonspecific binding of the probe to irrelevant chromosomes. Such an approach should improve the signal to noise ratio by 20-fold.

## Resolution power of mapping techniques

The resolution achievable by the subchromosomal mapping procedures described above lies in the range of 5–10 centimorgans. This estimate is based on the following considerations. Modern methods of chromosome banding permit the resolution of 850 bands per haploid genome<sup>34</sup>. Refinement of these methods can be expected to increase the band number to somewhat more than 1,000 (ref. 35). The total length of the human genome calculated in recombination units is about 3,000 centimorgans (ref. 36). Therefore, the number of bands is roughly one-third the number of centimorgans. Also relevant to the following discussion is the fact that the total human haploid nucleotide base pair number is about  $3 \times 10^9$ , and therefore there are an average of  $10^5$  base pairs (1 Mbp) per centimorgan.

Conventional low-resolution somatic cell genetic procedures can be expected to map genes to chromosomes and then within chromosomes to a resolution of 5–10 centimorgans. At the other end of the scale, high-resolution recombinant DNA procedures together with restriction site and nucleotide sequence analysis can be expected to provide mapping data from the level of the single nucleotide base pairs to the 100-kb level. These distances can be spanned by 'walking' along a chromosome from an identified cloned genomic DNA fragment using recombinant DNA techniques. This method involves isolating a DNA fragment within a genetically defined region by recombinant DNA

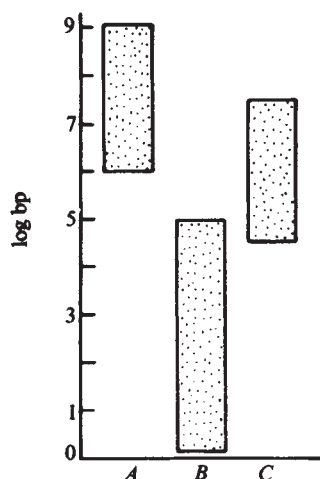
cloning from which the walk is started. This fragment is then used to probe a genomic library to identify clones which share a region of homology, but which extend beyond the initial fragment. These clones are then isolated, the process repeated and the linear nucleotide sequence extended. The 'walk' method combined with detailed cytogenetic maps has been used to advantage in *Drosophila*. However, *Drosophila* is especially well suited to this approach because there are relatively few redundant sequences which introduce false branch points in the walk, and because of the availability of the polytene *in situ* hybridization technique which provides a means of verifying progress along the chromosome (W. Bender, personal communication). However, mammalian genomes do not have these advantages.

Thus, in mammalian genetic linkage analysis a resolution 'gap' in the gene mapping techniques exists over the range 0.1–5.0 centimorgans (0.1–5.0 Mbp) (Fig. 2). This is a significant gap, because many functionally related clusters of genes, such as the human major histocompatibility locus, will fit into a space of this dimension. What mapping strategies might be devised to fill this gap?

Gene transfer systems provide possible approaches to intermediate level resolution analysis. All these methods depend on fragmenting linkage groups into small bits. In addition, all require a selectable marker in the linkage group of interest. The frequency of recovery of genes in linkage with the selectable marker provides information on the distance and order of genes with respect to the selectable marker. However, the requirement for a selectable marker has become less of a problem with the advent of DNA-mediated gene transfer. It is now possible to integrate various selectable markers into any linkage group by this procedure.

The three principal gene transfer systems suitable for intermediate level resolution analysis are: (1) DNA-mediated gene transfer, (2) donor chromosome breakage by irradiation and subsequent transfer to recipient cells by hybrid formation and (3) transfer of isolated metaphase chromosomes. The first system offers little advantage over walking, as the maximum fragment size is ~50 kb. The second method has already been successfully applied to linkage analysis by Goss and Harris<sup>37</sup>, who estimated the distance between the human genes thymidine kinase and galactose kinase to be 1.2 centimorgans by this technique<sup>38</sup>. The third method appears to have considerable potential for intermediate level resolution analysis, because a broad range of fragment sizes are generated by this procedure<sup>11</sup>. It has been shown that the procedure separates thymidine kinase and galactose kinase genes in 80% of transformants<sup>39</sup>, suggesting a potential for resolving genes separated by distances of 0.1 to possibly 0.05 centimorgans.

Klobutcher and Ruddle have recently introduced a breakage-translocation based approach to middle-resolution gene



**Fig. 2** Genetic mapping resolution by different methods. *a*, Gene mapping by somatic cell genetics; *b*, molecular genetic techniques; *c*, gene transfer and breakage-translocation methods. Ordinate = logarithm of base pair number.

mapping<sup>40</sup>. In this system, a donor chromosome is transferred into a recipient cell by one of the gene transfer techniques. The chromosome must carry a selectable marker which confers viability on the recipient cell when grown in selective conditions. An example would be the transfer of the thymidine kinase chromosome into a thymidine kinase-deficient recipient cell grown under hypoxanthine/aminopterin/thymidine selection. A second requirement is that the donor chromosome be lost (segregated) from the recipient cell at a frequency of ~3–10% of cells per generation. A translocation between the donor chromosome and a recipient chromosome can give rise to an interspecific recombinant chromosome which is stably retained, and which carries the donor selectable gene and the recipient centromere (Fig. 3). Even though such translocation events are rare, the resulting hybrid cell possesses a 3–10% per generation growth rate advantage, and will rapidly come to constitute an appreciable fraction of the original population.

Independently arising recombinants of this type can be isolated and then the order and relative distances between genes in the chromosome segment lying between the selectable marker and the centromere can be analysed. In the first application of this approach, recombinants were obtained in which the human selectable marker thymidine kinase and the closely linked human constitutive marker galactonkinase were separated with a dissociation frequency of ~40% which indicates that resolution at the 0.1 centimorgan level can be achieved. The break-translocation system has several advantages: (1) a single well characterized cell line is used which continuously generates recombinant sports; (2) chromosome breaks are selected only within a well defined region determined by the donor centromere and the prototrophic, donor marker; (3) in primate/rodent combinations, appropriate chromosome staining techniques can be used which permit the detection of the chromosome break points and allow a cytological estimation of the size of the translocated donor fragment.

Thus, it can be seen that a variety of gene transfer procedures have potential for establishing the order of and distance between genes within the intermediate range of resolution. These techniques can be subjected to rigorous evaluation only when numerous unique nucleic acid probes have been isolated which map into appropriately short segments of mammalian genomes amenable to analysis.

### Cooperative interaction between mendelian and somatic cell genetics

In 1911 the first sex-linked gene (colour blindness) was mapped in man<sup>41</sup>. It was not until 1970 that the first human autosomal gene was mapped using a strictly mendelian approach. However, since 1970, around 50 additional autosomal genes

have been mapped in man using family analysis. This significant increase in the accession of autosomal genes controlling complex traits can be attributed in many cases to the previous chromosome assignment of genes by somatic cell genetics. The Rh gene, first identified in 1939<sup>42</sup>, is a good case in point. In 1971, its linkage to Pep C was recognized<sup>43</sup>, and when in 1972 Pep C was assigned to chromosome 1 by somatic cell genetics, the Rh gene was also assigned<sup>44</sup>. In many instances, the chromosome assignment of a mutant or polymorphic trait segregating in families has been facilitated by a linkage association with a second gene which had been assigned previously by somatic cell genetics. This is a promising development, as more than 1,000 unassigned human genes have been described and catalogued, many of which produce genetic disease<sup>44</sup>.

One can expect the assignment of complex organismal traits to chromosomes to be accelerated through the mapping of restriction fragments by somatic cell genetics. Restriction fragment mapping is accomplished by Southern blotting analysis, hybridizing labelled fragments to DNA preparations derived from interspecific somatic cell hybrids carrying one or a few donor chromosomes, assuming there are differences between the lengths of the homologous fragments of the donor and recipient species. Subsequently, linkage tests or *in situ* hybridization mapping would provide a regional assignment within a particular chromosome for a specific sequence. If several hundred restriction fragment markers selected randomly were mapped, they would be spaced at approximately 5–10 centimorgan intervals. It can be argued on theoretical grounds that such restriction fragment markers will be highly polymorphic within the human species<sup>46</sup>. Wyman and White<sup>47</sup> have reported one restriction fragment length polymorphism in man which fits this description. DeMartinvill *et al.*<sup>48</sup> have recently mapped this marker to human chromosome 14. Co-segregation within a family of a complex trait (a trait expressed only at the organismal level) and a specific restriction fragment polymorphism, previously assigned to a specific chromosome, would allow that trait to be mapped.

In practice, this approach to human gene mapping could be carried out in the following way. DNA samples can be collected from members of families segregating genes which control the expression of complex traits or disease conditions, analysed for restriction fragment polymorphisms, and the resulting polymorphisms tested for linkage with other co-segregating traits. In this way, a variety of genes amenable only to mendelian analysis can be assigned positions on the human gene map. Using this approach, it should be feasible eventually to map the chromosomal locations of all the major genetic disease genes—for example, Huntington's chorea, cystic fibrosis and xeroderma pigmentosum. One of the practical advantages of the restriction site polymorphism mapping technique is that a single methodological procedure can be used to study many genetic polymorphisms within a single kindred. In this manner, the restriction fragment length polymorphism method is similar to the standard practice of using serological tests for studying linkage relationships in human families.

Several techniques are available for the isolation of cloned DNA probes whose corresponding homologous sequences can be mapped. Some of these probes will represent sequences within genes of known function (for example, the globins or the insulins), while others will be of unknown function when first isolated. Fragments of both known and unknown function can be mapped and used as genetic markers equally well. To serve as discrete markers, the cloned fragments (probes) should be unique, single copy sequences. These can be obtained conveniently in cDNA libraries<sup>49</sup>. The cDNA probes have the additional advantage that they represent structural gene products and their biological function will ultimately become known. The cDNA probes will also be characterized to a degree by their tissues of origin. It is also possible to isolate DNA probes from genomic libraries, but in these instances the probability of recovering redundant sequences is significantly increased. Genomic sequences may, however, be subcloned,



subfragments tested for redundancy, and the desired unique sequences isolated.

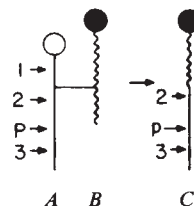
Once a gene controlling a complex trait or genetic disease is mapped to a chromosomal region, additional restriction fragment markers can be mapped into that region using somatic cell genetics techniques. For example, Gusella *et al.*<sup>50</sup> have produced cloned gene libraries from a man  $\times$  mouse hybrid cell population carrying human chromosome 11 as a single donor chromosome. Such a library would contain fragments predominantly from the total mouse genome and a smaller set of clones containing DNA fragments from human chromosome 11. A subset of the human fragment clones will also contain repetitive human DNA species which can be detected by colony hybridization with labelled total human DNA. Gusella showed that in many instances the human repetitive sequences were contiguous with unique sequences. The single copy sequences could be isolated and then used as unique probes for the detection of their corresponding homologous sequences within chromosome 11. These were shown by direct analysis to map to chromosome 11 as expected. Subsequent regional mapping experiments assigned four such fragments to different regions of the chromosome. Thus, it is possible, using a cell hybrid which retains a defined donor genomic segment, to isolate cloned gene fragments which map specifically to that segment.

## Application of gene mapping to prenatal diagnosis

The ability to map restriction fragment markers randomly throughout the genome and to specific regions of chromosomes has obvious important implications for prenatal diagnosis, genetic screening of populations and paternity testing. Restriction fragment length polymorphisms closely linked to mutant alleles have already been used to predict genetic disease prenatally. In the case of sickle cell anaemia, Kan and Dozy have reported a polymorphism at a *Hpa*I site 5,000 bp distant from the 3' end of the  $\alpha$ -globin gene<sup>51</sup>. When this site is present, a 7-kb fragment detectable with  $\alpha$ -globin probes is generated; when absent, a 13-kb fragment is produced. The *Hpa*I site, which is responsible for the restriction fragment length polymorphism, is tightly linked to the  $\alpha$ -globin locus (5,000 bp is equivalent to 0.005 centimorgans) so that the probability of recombination between the *Hpa*I site and the  $\alpha$ -globin locus is essentially zero. Therefore, if the polymorphism is present in heterozygous conditions in a family at risk for sickle cell disease, and if appropriate information exists to establish the linkage relationships between the polymorphic site and the  $\alpha$ -globin alleles in the parents, the restriction fragment polymorphism can be used to predict the  $\alpha$ -globin genotype of the fetus.

Kan and Dozy have calculated that 60% of black families in North America at risk for sickle cell anaemia are amenable to this kind of predictive analysis. Other polymorphisms in or around the  $\alpha$ -globin locus can be used for the same purpose, and serve to increase the number of families which might benefit from the procedure<sup>52</sup>. Geever *et al.*<sup>53</sup> have recently reported a restriction site change which is directly related to the nucleotide base substitution responsible for the conversion of the wild-type  $\alpha$ -globin gene to the sickle cell anaemia mutated version. In this case, there is a one to one relationship between the restriction pattern and mutant gene, thereby obviating the necessity of testing for linked polymorphisms in family members. Such direct tests for the presence or absence of gene mutations are likely to be the preferred prenatal diagnostic tests of the future.

The  $\alpha$ -globin example must not be taken as a representative case. The biochemical function of the  $\alpha$ -globin complex is known and more than 100 kb of the  $\alpha$ -globin region has been cloned. On the other hand, the biochemical lesions of many disease genes are unknown, and strategies for cloning these genes have not yet been devised. It should be possible to establish linkage between such a disease gene and a restriction fragment marker mendelian mapping, but finding tight linkage relationships of 1 centimorgan or less will be time consuming



**Fig. 3** Breakage-translocation method of establishing gene linkage relationships. A, donor chromosome; B, recipient chromosome; C, translocated chromosome resulting from exchange between the donor and recipient chromosomes. 1-3, Constitutive genes; P, prototrophic gene. The reciprocal rearrangement product bearing the donor chromosome is presumed to be lost.

and costly. On the other hand, a linkage distance in excess of 1 centimorgan is unlikely to be acceptable for prenatal diagnosis, so that in many instances cloning of the disease gene may be a necessary step in developing a prenatal diagnostic test based on the detection of restriction fragment polymorphisms. Alternatively, predictive accuracy can be significantly improved by mapping restriction fragment markers to positions flanking the disease gene. Positive identification of a genetically diseased fetus would then depend on the parental configuration of the outside markers (the non-cross-over class). False negative predictions for the disease gene would in this case result from double cross-overs occurring in the intervals flanking it. However, the error frequency will be small and is a function of the size of the flanking genetic intervals. If the distance between the disease gene and each of the flanking markers was 1 centimorgan the probability of the double cross-over would be 0.0001. The mapping of multiple restriction fragment markers in the vicinity of a disease gene can be accomplished by means of somatic cell genetic procedures referred to above. The availability of numerous, closely linked, flanking markers would increase the probability of detecting the appropriately polymorphic combinations within any given family. Moreover, somatic cell genetic procedures can be used to determine the *cis/trans* relationship of flanking markers within particular family members.

This article is dedicated to the memory of Mary Lindley. I thank my colleagues for criticisms and suggestions, and Marie Siniscalchi for preparation of the typescript. Much of this work has been supported by NIH grant GM09966.

- Harris, M. in *Cell Culture and Somatic Variation* (Holt, Rinehart & Winston, New York, 1964).
- Ringertz, N. & Savage, R. in *Cell Hybrids* (Academic, New York, 1976).
- Siminovich, L. *Cell* **7**, 1-11 (1976).
- Kao, F., Chasin, L. & Puck, T. *Proc. natn. Acad. Sci. U.S.A.* **64**, 1284-1291 (1969).
- Davidson, R. L. *A. Rev. Genet.* **8**, 195-218 (1974).
- Ruddle, F. H. & Creagan, R. P. *A. Rev. Genet.* **9**, 407-486 (1975).
- McKusick, V. & Ruddle, F. *Science* **196**, 390-405 (1977).
- Fournier, R. E. K. & Ruddle, F. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 319-323 (1977).
- Klobutcher, L. A. & Ruddle, F. H. *A. Rev. Biochem.* **50**, 533-554 (1981).
- McBride, O. W. & Ozer, H. L. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1258-1262 (1973).
- Miller, C. L. & Ruddle, R. H. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3346-3350 (1978).
- Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, F., Cheng, Y.-C. & Axel, R. *Cell* **11**, 223-232 (1977).
- Capocchi, M. *Cell* **22**, 479-488 (1980).
- Ruddle, F. H. in *Genes, Chromosomes and Neoplasia* (eds Arrighi, F. E., Rao, P. N. & Stubblefield, E.) (Ravel, New York, 1981).
- Deisseroth, A. *et al. Cell* **12**, 205-218 (1977).
- Deisseroth, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 1456-1460 (1978).
- Swan, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 2735-2739 (1979).
- D'Eustachio, P., Bothwell, A. L., Takaro, T. T., Baltimore, D. & Ruddle, F. H. *J. exp. Med.* **153**, 793-800 (1981).
- D'Eustachio, P., Pravtcheva, D., Marcu, K. & Ruddle, F. H. *J. exp. Med.* **151**, 1545-1550 (1980).
- Ricciuti, F. & Ruddle, F. H. *Genetics* **74**, 661-678 (1973).
- The Human Genetic Mutant Repository* (NIH Publ. 80-2011, 1980).
- Bergsma, D. *Human Gene Mapping Vols I (1973), II (1974), III (1976), IV (1978), V (1979)*.
- Gusella, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 5239-5243 (1979).
- Gray, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 1231-1234 (1975).
- Lebo, R. V. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 5804-5808 (1979).
- Gall, J. & Pardue, M. L. *Proc. natn. Acad. Sci. U.S.A.* **63**, 378-383 (1969).
- Robins, D., Ripley, S., Henderson, A. & Axel, R. *Cell* **23**, 29-40 (1981).
- Malcolm, S., Barton, P., Murphy, C. & Ferguson-Smith, M. *Ann. Hum. Genet.* **45**, 135-142 (1981).
- Chandler-Harper, M. E. & Saunders, G. *Chromosoma* (in the press).
- Harper, M., Ullrich, A. & Saunders, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4458-4460 (1981).
- Harper, M., Barrera-Saldana, H. & Saunders, G. *Am. J. hum. Genet.* (in the press).

32. Wahl, G., Stern, M. & Stark, G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683–3687 (1979).
33. Diaz, M., Barsacchi-Pilone, G., Mahon, K. & Gall, J. *Cell* **24**, 649–659 (1981).
34. Yunis, J. *Hum. Genet.* **56**, 293–298 (1981).
35. Yunis, J., Sawyer, J. & Ball, D. *Chromosoma* **67**, 293–307 (1978).
36. Renwick, J. *Br. med. Bull.* **25**, 65–73 (1969).
37. Goss, S. J. & Harris, H. *Nature* **255**, 680–684 (1975).
38. Goss, S. *Cytogenet. Cell Genet.* **25**, 1–4 (1979).
39. Ruddle, F. H. & McBride, W. in *Molecular Biology of the Mammalian Genetic Apparatus* (Elsevier, Amsterdam, 1977).
40. Klobutcher, L. A. & Ruddle, F. H. *Nature* **280**, 657–660 (1979).
41. Wilson, E. B. *Arch. Mikrosk. Anat.* **77**, 249–271 (1911).
42. Levine, P. & Stetson, R. E. *J. Am. med. Ass.* **113**, 126–127 (1939).
43. Van Cong, N., Billardon, C., Picard, J., Feingold, J. & Frézal, J. *Cr. hebd. Séanc. Acad. Sci. Paris* **272**, 485 (1971).
44. Ruddle, F. H. *et al. Science* **176**, 1429–1431 (1972).
45. McKusick, V. A. in *Mendelian Inheritance in Man* (Johns Hopkins University Press, Baltimore, 1978).
46. Botstein, D., White, R., Skolnick, M. & Davis, R. *Am. J. hum. Genet.* **32**, 314–331 (1980).
47. Wyman, A. & White, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6754–6758 (1980).
48. DeMartinville, B., Wyman, A., White, R. & Francke, U. *Pediat. Res.* **15**, 560 (1981).
49. Woods, D., Crampton, J., Clarke, B. & Williamson, R. *Nucleic Acids Res.* **8**, 5157–5168 (1980).
50. Gusella, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 2829–2833 (1980).
51. Kan, Y. & Dozy, A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5631–5635 (1978).
52. Phillips, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 2853–2856 (1980).
53. Geever, R. *et al. Proc. natn. Acad. Sci. U.S.A.* (in the press).
54. Owerbach, D. *et al. Somatic Cell Genet.* **7**, 359–369 (1981).
55. Owerbach, D., Rutter, W., Cooke, N., Martial, J. & Shows, T. *Science* **212**, 815–816 (1981).
56. Owerbach, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 3123–3127 (1981).
57. Owerbach, D., Bell, G., Rutter, W. & Shows, T. *Nature* **286**, 82–84 (1980).
58. Harper, M. & Sanders, G. *Eur. J. Cell Biol.* **22**, 20 (1980).
59. Owerbach, D., Rutter, W., Martial, J., Baxter, J. & Shows, T. *Science* **209**, 289–292 (1980).
60. George, D., Phillips, J., Francke, U. & Seeburg, P. *Hum. Genet.* **57**, 138–141 (1981).
61. Wolf, S., Mareni, C. & Migeon, B. *Cell* **21**, 95–102 (1980).
62. D'Eustachio, P., Meyuhas, O., Ruddle, F. H. & Perry, R. P. *Cell* **24**, 307–312 (1981).
63. Yagi, M., D'Eustachio, P., Ruddle, F. H. & Koshland, M. J. *exp Med.* (in the press).
64. Roberts, M., Huttner, K. M., Schimke, R. T. & Ruddle, F. H. *J. Cell Biol.* **87**, SG2211 (1980).
65. D'Eustachio, P., Ingram, P. S., Tilghman, S. M. & Ruddle, F. H. *Somatic Cell Genet.* **7**, 289–294 (1981).
66. Leder, A., Swan, D., Ruddle, F., D'Eustachio, P. & Leder, P. *Nature* **293**, 196–200 (1981).
67. Gupta, P., D'Eustachio, P., Ruddle, F. & Rosen, J. *J. Cell Biol.* **87**, D841 (1980).

## ARTICLES

# <sup>40</sup>Ar/<sup>39</sup>Ar age spectra from the KBS Tuff, Koobi Fora Formation

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<sup>40</sup>Ar/<sup>39</sup>Ar age spectra on anorthoclase phenocrysts from three pumice clasts in the KBS Tuff yield nearly ideal flat patterns, providing good evidence that the samples have remained undisturbed since crystallization. The ages are concordant at  $1.88 \pm 0.02$  Myr, and confirm that the KBS Tuff, a key marker bed in the Koobi Fora Formation, northern Kenya, is now very well dated. These results resolve the conflict between earlier <sup>40</sup>Ar/<sup>39</sup>Ar and conventional K–Ar dating measurements on the KBS Tuff.

THE KBS Tuff is an important marker bed in the Pliocene to Pleistocene sedimentary sequence deposited in a basin centred on lat. 4° N, long. 36.3° E, adjacent to the eastern shores of Lake Turkana, northern Kenya<sup>1–3</sup>. Vertebrate fossils, including hominids, have been found<sup>4–9</sup> in stratigraphic proximity to the KBS Tuff, at the base of which numerous stone tools have been recovered<sup>6</sup>. Thus it is of interest to establish accurately the age of the KBS Tuff, especially in regard to hominid evolution<sup>10</sup>. There has been much controversy as to the age of this bed<sup>7–9,11–13</sup>, but recent dating studies on minerals separated from pumice clasts found within the KBS Tuff, have provided relatively consistent ages within the range  $1.85 \pm 0.1$  Myr<sup>13–15</sup>. The most precise age estimate of  $1.89 \pm 0.01$  Myr was based on conventional K–Ar isotopic age measurements on anorthoclase feldspar<sup>13</sup>.

An unresolved problem concerns understanding the meaning of the ages for the KBS Tuff reported by Fitch, Miller and coworkers<sup>11,16–19</sup>, who mainly used the <sup>40</sup>Ar/<sup>39</sup>Ar dating technique on anorthoclase separated from pumice clasts. The <sup>40</sup>Ar/<sup>39</sup>Ar total fusion ages ranged from  $0.52 \pm 0.33$  to  $2.6 \pm 0.3$  Myr, and the step heating measurements yielded complex age spectra, interpreted<sup>11,17–19</sup> as indicating marked disturbance of the anorthoclase subsequent to its crystallization. They suggested that crystallization occurred ~2.48 Myr ago, with deposition in the KBS Tuff shortly thereafter, followed by thermal overprinting at various times, especially at ~1.8 and 1 Myr ago.

Here I present results of dating of anorthoclase separated from three pumice clasts found within the KBS Tuff, using the <sup>40</sup>Ar/<sup>39</sup>Ar total fusion and age spectrum techniques. These data provide strong evidence that the samples have remained undisturbed since crystallization, which occurred  $1.88 \pm 0.02$  Myr ago.

## KBS Tuff

The KBS Tuff occurs within an essentially flatlying sequence of sediments, little more than 300-m thick, that crops out adjacent to Lake Turkana over an area at least 80 km (north–south) by 30 km (east–west). The KBS Tuff, commonly ~1-m thick, is the topmost unit of the lower member of the Koobi Fora Formation, and is overlain by no more than 125 m of sediment<sup>1–3</sup>. In common with other tuffs in the sequence, the KBS Tuff was transported by and deposited from water<sup>3</sup>, and consists mainly of rhyolitic glass with some admixture of early Palaeozoic and Tertiary detrital material.

Locally within the KBS Tuff, pumice clasts are found, regarded as products of the same volcanic eruptions that produced the bulk of the tuff<sup>3</sup>. Pumice clasts are used for the isotopic dating because they are less likely to be contaminated by old detrital material compared with the enclosing tuff. Here I have used anorthoclase separated from three pumice clasts, previously dated by the conventional K–Ar method<sup>13</sup>. Sample 78-1038 is from the KBS Tuff in area 105 (grid ref. HBH ~ 730497), adjacent to its type locality, whereas samples 78-1047 and 79-14 are from a correlative of the KBS Tuff in area 131 (grid ref. HBH ~ 774594), about 10 km north-northeast of the locality for sample 78-1038. Geochemical data<sup>20</sup> from pumice clasts confirm that it is the same tuff in areas 105 and 131, but that the tuff in area 112 which yielded<sup>13</sup> similar K–Ar ages is not likely to be the same bed as the type KBS Tuff.

## Methods

In the <sup>40</sup>Ar/<sup>39</sup>Ar dating method, <sup>39</sup>Ar is generated from <sup>39</sup>K in the sample by fast neutrons in a nuclear reactor, following which Ar is extracted in a high vacuum system and analysed isotopically, allowing simultaneous determination of K and



**Table 1**  $^{40}\text{Ar}/^{39}\text{Ar}$  isotopic data and ages on anorthoclase from pumice clasts, KBS Tuff, Koobi Fora Formation, Kenya

| Temperature<br>(°C)                                                                                                    | $^{39}\text{Ar}$<br>( $10^{-15}$ mol) | $^{37}\text{Ar}$<br>( $10^{-16}$ mol) | $^{36}\text{Ar}$<br>( $10^{-17}$ mol) | $^{40}\text{Ar}$<br>( $10^{-15}$ mol) | (100 Rad $^{40}\text{Ar}$ )/<br>(Total $^{40}\text{Ar}$ ) | Calculated age<br>(Myr) |
|------------------------------------------------------------------------------------------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|-----------------------------------------------------------|-------------------------|
| <b>78-1038 (KF38) Anorthoclase, step heat 1, 210–350 <math>\mu\text{m}</math>, 1.240 g, <math>J = 0.000646</math></b>  |                                       |                                       |                                       |                                       |                                                           |                         |
| 600                                                                                                                    | 46 $\pm$ 1                            | 127 $\pm$ 80                          | 329 $\pm$ 10                          | 1,063 $\pm$ 1                         | 8.7                                                       | 2.315 $\pm$ 0.714       |
| 700                                                                                                                    | 230 $\pm$ 1                           | 128 $\pm$ 53                          | 185 $\pm$ 5                           | 951 $\pm$ 1                           | 42.3                                                      | 2.037 $\pm$ 0.069       |
| 770                                                                                                                    | 474 $\pm$ 1                           | —                                     | 156 $\pm$ 6                           | 1,271 $\pm$ 1                         | 62.9                                                      | 1.967 $\pm$ 0.046       |
| 820                                                                                                                    | 627 $\pm$ 1                           | 222 $\pm$ 50                          | 134 $\pm$ 6                           | 1,426 $\pm$ 1                         | 71.4                                                      | 1.892 $\pm$ 0.035       |
| 870                                                                                                                    | 795 $\pm$ 1                           | —                                     | 130 $\pm$ 3                           | 1,686 $\pm$ 1                         | 76.3                                                      | 1.884 $\pm$ 0.018       |
| 915                                                                                                                    | 801 $\pm$ 1                           | 128 $\pm$ 128                         | 129 $\pm$ 6                           | 1,652 $\pm$ 2                         | 76.1                                                      | 1.830 $\pm$ 0.029       |
| 940                                                                                                                    | 984 $\pm$ 1                           | 306 $\pm$ 76                          | 139 $\pm$ 4                           | 1,977 $\pm$ 1                         | 78.5                                                      | 1.836 $\pm$ 0.017       |
| 970                                                                                                                    | 859 $\pm$ 1                           | 104 $\pm$ 92                          | 114 $\pm$ 1                           | 1,728 $\pm$ 1                         | 79.6                                                      | 1.867 $\pm$ 0.011       |
| 1,000                                                                                                                  | 1,165 $\pm$ 2                         | 49 $\pm$ 86                           | 128 $\pm$ 6                           | 2,274 $\pm$ 1                         | 82.4                                                      | 1.873 $\pm$ 0.020       |
| 1,040                                                                                                                  | 1,326 $\pm$ 1                         | 187 $\pm$ 93                          | 126 $\pm$ 3                           | 2,533 $\pm$ 2                         | 84.3                                                      | 1.877 $\pm$ 0.013       |
| 1,070                                                                                                                  | 1,236 $\pm$ 2                         | 259 $\pm$ 66                          | 124 $\pm$ 6                           | 2,378 $\pm$ 4                         | 83.7                                                      | 1.876 $\pm$ 0.021       |
| 1,110                                                                                                                  | 1,197 $\pm$ 1                         | 121 $\pm$ 280                         | 107 $\pm$ 4                           | 2,308 $\pm$ 1                         | 85.3                                                      | 1.915 $\pm$ 0.015       |
| 1,160                                                                                                                  | ( $\sim$ 910)                         | gas lost                              | —                                     | —                                     | —                                                         | —                       |
| 1,225                                                                                                                  | 1,389 $\pm$ 2                         | —                                     | 147 $\pm$ 5                           | 2,720 $\pm$ 4                         | 83.1                                                      | 1.894 $\pm$ 0.017       |
| 1,360                                                                                                                  | 782 $\pm$ 1                           | 130 $\pm$ 108                         | 521 $\pm$ 9                           | 2,850 $\pm$ 2                         | 45.5                                                      | 1.931 $\pm$ 0.040       |
| 1,470                                                                                                                  | 74 $\pm$ 1                            | 402 $\pm$ 36                          | 137 $\pm$ 3                           | 540 $\pm$ 1                           | 25.3                                                      | 2.153 $\pm$ 0.155       |
| Total                                                                                                                  | 11,985                                | 2,163                                 | 2,606                                 | 27,357                                | —                                                         | 1.89 $\pm$ 0.03         |
| <b>78-1038 (KF38) Anorthoclase, step heat 2, 210–350 <math>\mu\text{m}</math>, 0.648 g, <math>J = 0.000644</math></b>  |                                       |                                       |                                       |                                       |                                                           |                         |
| 760                                                                                                                    | 118 $\pm$ 1                           | 193 $\pm$ 70                          | 295 $\pm$ 2                           | 1,088 $\pm$ 1                         | 20.0                                                      | 2.149 $\pm$ 0.061       |
| 830                                                                                                                    | 302 $\pm$ 1                           | 113 $\pm$ 18                          | 144 $\pm$ 4                           | 911 $\pm$ 1                           | 52.9                                                      | 1.856 $\pm$ 0.043       |
| 890                                                                                                                    | 408 $\pm$ 1                           | 64 $\pm$ 145                          | 93 $\pm$ 6                            | 938 $\pm$ 1                           | 69.8                                                      | 1.864 $\pm$ 0.052       |
| 930                                                                                                                    | 442 $\pm$ 1                           | 64 $\pm$ 134                          | 90 $\pm$ 4                            | 966 $\pm$ 1                           | 71.7                                                      | 1.816 $\pm$ 0.030       |
| 970                                                                                                                    | 580 $\pm$ 1                           | —                                     | 87 $\pm$ 3                            | 1,212 $\pm$ 1                         | 77.9                                                      | 1.892 $\pm$ 0.020       |
| 1,000                                                                                                                  | 548 $\pm$ 1                           | 152 $\pm$ 129                         | 93 $\pm$ 4                            | 1,165 $\pm$ 1                         | 75.8                                                      | 1.868 $\pm$ 0.026       |
| 1,030                                                                                                                  | 590 $\pm$ 1                           | 142 $\pm$ 66                          | 76 $\pm$ 5                            | 1,203 $\pm$ 1                         | 80.4                                                      | 1.906 $\pm$ 0.028       |
| 1,065                                                                                                                  | 610 $\pm$ 1                           | 131 $\pm$ 49                          | 70 $\pm$ 4                            | 1,208 $\pm$ 1                         | 82.1                                                      | 1.887 $\pm$ 0.027       |
| 1,100                                                                                                                  | 751 $\pm$ 1                           | 73 $\pm$ 111                          | 160 $\pm$ 2                           | 1,686 $\pm$ 1                         | 71.2                                                      | 1.855 $\pm$ 0.012       |
| 1,150                                                                                                                  | 849 $\pm$ 1                           | 57 $\pm$ 139                          | 122 $\pm$ 5                           | 1,749 $\pm$ 1                         | 78.5                                                      | 1.878 $\pm$ 0.023       |
| 1,200                                                                                                                  | 1,188 $\pm$ 1                         | 201 $\pm$ 98                          | 142 $\pm$ 4                           | 2,369 $\pm$ 1                         | 81.5                                                      | 1.885 $\pm$ 0.015       |
| 1,430                                                                                                                  | 468 $\pm$ 1                           | 326 $\pm$ 178                         | 295 $\pm$ 3                           | 1,640 $\pm$ 3                         | 46.4                                                      | 1.887 $\pm$ 0.027       |
| Total                                                                                                                  | 6,854                                 | 1,516                                 | 1,667                                 | 16,135                                | —                                                         | 1.88 $\pm$ 0.03         |
| <b>78-1038 (KF38) Anorthoclase, total fusion, 0.143 g, <math>J = 0.000646</math></b>                                   |                                       |                                       |                                       |                                       |                                                           |                         |
| 1,500                                                                                                                  | 1,413 $\pm$ 1                         | 130 $\pm$ 49                          | 257 $\pm$ 4                           | 3,089 $\pm$ 1                         | 74.6                                                      | 1.900 $\pm$ 0.014       |
| <b>78-1047 (KF47) Anorthoclase, step heat, 210–350 <math>\mu\text{m}</math>, 1.367 g, <math>J = 0.000658</math></b>    |                                       |                                       |                                       |                                       |                                                           |                         |
| 600                                                                                                                    | 34 $\pm$ 1                            | 8 $\pm$ 8                             | 584 $\pm$ 6                           | 1,779 $\pm$ 2                         | 3.0                                                       | 1.846 $\pm$ 0.553       |
| 720                                                                                                                    | 32 $\pm$ 1                            | —                                     | 173 $\pm$ 2                           | 547 $\pm$ 1                           | 6.5                                                       | 1.321 $\pm$ 0.909       |
| 790                                                                                                                    | 500 $\pm$ 1                           | 56 $\pm$ 20                           | 618 $\pm$ 6                           | 2,666 $\pm$ 3                         | 31.2                                                      | 1.977 $\pm$ 0.057       |
| 850                                                                                                                    | 771 $\pm$ 1                           | 112 $\pm$ 11                          | 594 $\pm$ 6                           | 2,995 $\pm$ 3                         | 41.0                                                      | 1.890 $\pm$ 0.035       |
| 900                                                                                                                    | 991 $\pm$ 1                           | 82 $\pm$ 11                           | 553 $\pm$ 8                           | 3,218 $\pm$ 3                         | 48.6                                                      | 1.874 $\pm$ 0.041       |
| 940                                                                                                                    | 1,179 $\pm$ 1                         | 107 $\pm$ 24                          | 419 $\pm$ 7                           | 3,125 $\pm$ 3                         | 59.8                                                      | 1.881 $\pm$ 0.024       |
| 970                                                                                                                    | 1,225 $\pm$ 1                         | 138 $\pm$ 11                          | 374 $\pm$ 3                           | 3,093 $\pm$ 3                         | 63.6                                                      | 1.907 $\pm$ 0.013       |
| 1,000                                                                                                                  | 1,280 $\pm$ 1                         | 135 $\pm$ 29                          | 399 $\pm$ 8                           | 3,208 $\pm$ 3                         | 62.6                                                      | 1.861 $\pm$ 0.023       |
| 1,045                                                                                                                  | 1,527 $\pm$ 2                         | 127 $\pm$ 25                          | 390 $\pm$ 5                           | 3,580 $\pm$ 4                         | 67.1                                                      | 1.866 $\pm$ 0.015       |
| 1,070                                                                                                                  | 1,551 $\pm$ 2                         | 115 $\pm$ 21                          | 371 $\pm$ 3                           | 3,602 $\pm$ 4                         | 68.8                                                      | 1.897 $\pm$ 0.013       |
| 1,130                                                                                                                  | 1,799 $\pm$ 2                         | 153 $\pm$ 23                          | 425 $\pm$ 6                           | 4,095 $\pm$ 4                         | 68.5                                                      | 1.852 $\pm$ 0.016       |
| 1,180                                                                                                                  | 1,851 $\pm$ 2                         | 144 $\pm$ 3                           | 453 $\pm$ 7                           | 4,292 $\pm$ 4                         | 68.0                                                      | 1.872 $\pm$ 0.016       |
| 1,210                                                                                                                  | ( $\sim$ 840)                         | gas lost                              | —                                     | —                                     | —                                                         | —                       |
| 1,250                                                                                                                  | 545 $\pm$ 1                           | 26 $\pm$ 20                           | 480 $\pm$ 5                           | 2,319 $\pm$ 2                         | 38.4                                                      | 1.938 $\pm$ 0.034       |
| 1,320                                                                                                                  | 133 $\pm$ 1                           | 10 $\pm$ 19                           | 1,052 $\pm$ 8                         | 3,297 $\pm$ 5                         | 5.6                                                       | 1.656 $\pm$ 0.229       |
| Total                                                                                                                  | 13,418                                | 1,213                                 | 6,885                                 | 41,816                                | —                                                         | 1.88 $\pm$ 0.03         |
| <b>78-1047 (KF47) Anorthoclase, total fusion, 0.625 g, <math>J = 0.000661</math></b>                                   |                                       |                                       |                                       |                                       |                                                           |                         |
| 1,500                                                                                                                  | 6,515 $\pm$ 6                         | 541 $\pm$ 26                          | 1,525 $\pm$ 8                         | 15,220 $\pm$ 7                        | 67.6                                                      | 1.881 $\pm$ 0.011       |
| <b>79-14 (7722-1071) Anorthoclase, step heat, 100–350 <math>\mu\text{m}</math>, 1.339 g, <math>J = 0.000568</math></b> |                                       |                                       |                                       |                                       |                                                           |                         |
| 600                                                                                                                    | 96 $\pm$ 1                            | —                                     | 575 $\pm$ 4                           | 1,934 $\pm$ 3                         | 12.0                                                      | 2.474 $\pm$ 0.155       |
| 700                                                                                                                    | 80 $\pm$ 1                            | 251 $\pm$ 175                         | 270 $\pm$ 8                           | 982 $\pm$ 1                           | 18.8                                                      | 2.355 $\pm$ 0.320       |
| 730                                                                                                                    | 116 $\pm$ 1                           | —                                     | 217 $\pm$ 10                          | 898 $\pm$ 1                           | 28.3                                                      | 2.241 $\pm$ 0.272       |
| 760                                                                                                                    | 274 $\pm$ 1                           | —                                     | 257 $\pm$ 9                           | 1,369 $\pm$ 1                         | 44.0                                                      | 2.252 $\pm$ 0.103       |
| 820                                                                                                                    | 1,259 $\pm$ 1                         | 352 $\pm$ 85                          | 538 $\pm$ 4                           | 4,005 $\pm$ 3                         | 59.5                                                      | 1.941 $\pm$ 0.023       |
| 880                                                                                                                    | 1,258 $\pm$ 2                         | —                                     | 377 $\pm$ 8                           | 3,463 $\pm$ 5                         | 66.9                                                      | 1.886 $\pm$ 0.028       |
| 930                                                                                                                    | 2,259 $\pm$ 2                         | —                                     | 466 $\pm$ 8                           | 5,557 $\pm$ 5                         | 74.1                                                      | 1.868 $\pm$ 0.022       |
| 965                                                                                                                    | 1,593 $\pm$ 1                         | —                                     | 274 $\pm$ 9                           | 3,770 $\pm$ 2                         | 77.4                                                      | 1.877 $\pm$ 0.026       |
| 1,000                                                                                                                  | 1,168 $\pm$ 1                         | 51 $\pm$ 84                           | 209 $\pm$ 1                           | 2,765 $\pm$ 3                         | 76.6                                                      | 1.858 $\pm$ 0.019       |
| 1,045                                                                                                                  | 1,139 $\pm$ 1                         | 141 $\pm$ 132                         | 197 $\pm$ 3                           | 2,704 $\pm$ 2                         | 77.4                                                      | 1.883 $\pm$ 0.021       |
| 1,100                                                                                                                  | 494 $\pm$ 1                           | —                                     | 106 $\pm$ 6                           | 1,250 $\pm$ 1                         | 73.8                                                      | 1.912 $\pm$ 0.043       |
| 1,150                                                                                                                  | 946 $\pm$ 1                           | 206 $\pm$ 127                         | 228 $\pm$ 9                           | 2,493 $\pm$ 1                         | 72.0                                                      | 1.942 $\pm$ 0.034       |
| 1,250                                                                                                                  | 997 $\pm$ 1                           | 187 $\pm$ 142                         | 557 $\pm$ 10                          | 3,736 $\pm$ 2                         | 55.2                                                      | 2.122 $\pm$ 0.037       |
| 1,400                                                                                                                  | 78 $\pm$ 1                            | 90 $\pm$ 110                          | 1,090 $\pm$ 7                         | 3,316 $\pm$ 3                         | 2.8                                                       | 1.226 $\pm$ 0.310       |
| Total                                                                                                                  | 11,757                                | 1,278                                 | 5,361                                 | 38,242                                | —                                                         | 1.92 $\pm$ 0.04         |
| <b>79-14 (7722-1071) Anorthoclase, total fusion, 0.454 g, <math>J = 0.000564</math></b>                                |                                       |                                       |                                       |                                       |                                                           |                         |
| 1,500                                                                                                                  | 4,122 $\pm$ 9                         | —                                     | 1,819 $\pm$ 1                         | 13,100 $\pm$ 30                       | 58.1                                                      | 1.879 $\pm$ 0.023       |

$\lambda = 5.543 \times 10^{-10} \text{ yr}^{-1}$ . Errors at level of 1 s.d. All data corrected for instrument discrimination.  $^{37}\text{Ar}$  and  $^{39}\text{Ar}$  corrected for decay. Line blanks have not been applied, but are as follows for  $^{40}\text{Ar}$ : 78-1038 step heat 1,  $2.5 \times 10^{-13}$  mol; 78-1038 step heat 2,  $1.5 \times 10^{-13}$  mol; 78-1047 step heat,  $4 \times 10^{-13}$  mol; 79-14 step heat,  $3 \times 10^{-13}$  mol. Reported absolute amounts have an additional untabulated uncertainty of  $\sim 10\%$ .

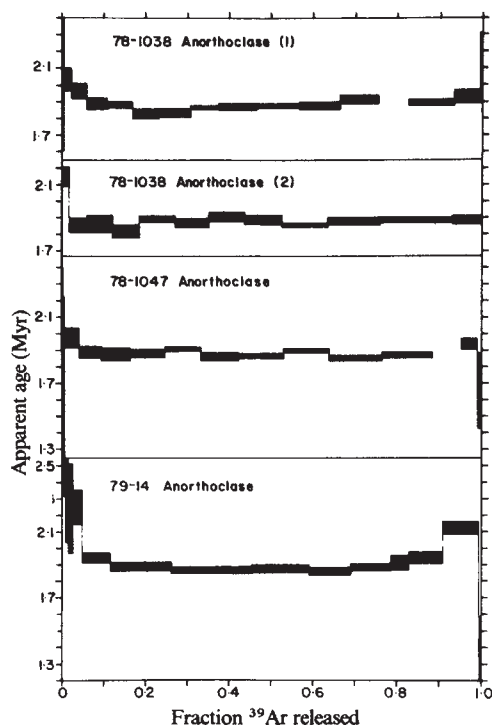


Fig. 1  $^{40}\text{Ar}/^{39}\text{Ar}$  age spectra for anorthoclase from pumice clasts in the KBS Tuff. Data plotted as apparent age against fraction of  $^{39}\text{Ar}$  released. Uncertainty of the age for each step, at the level of 1 s.d., indicated by thickness of bar.

radiogenic Ar ( $^{40}\text{Ar}^*$ ) (ref. 21). The ratio  $^{40}\text{Ar}^*/^{39}\text{Ar}$  is proportional to age. The method is applied in two distinct ways, both of which are used here. First, the sample can be fused after irradiation and from the measured isotopic composition of the Ar an age can be derived, which normally agrees with the conventional K–Ar age. Second, after irradiation of the sample, the Ar can be released in stages by stepwise or incremental heating, commencing at a temperature well below that of fusion, and the gas evolved in successive steps analysed to provide a series of apparent ages. A plot of age versus fraction of  $^{39}\text{Ar}$  released, an age spectrum, may be interpreted<sup>22</sup> in terms of distribution of  $^{40}\text{Ar}^*$  and  $^{39}\text{Ar}$  in the sample.

Anorthoclase should be nearly ideal for  $^{40}\text{Ar}/^{39}\text{Ar}$  age spectrum measurements as it remains stable in the vacuum system during much of the heating, so that diffusion is likely to be the major transport mechanism for extraction of Ar. The crystal fragments were kept relatively coarse, in the range 100–350  $\mu\text{m}$ , in the hope that if geologically-induced diffusion profiles for  $^{40}\text{Ar}^*$  are present, they would be revealed by the age spectrum measurements.

Irradiations for 30 h were carried out in a horizontal facility adjacent to, but not within the core of Hifar reactor<sup>23,24</sup>, where the fast neutron flux is  $\sim 4 \times 10^{12}$  neutrons  $\text{cm}^{-2} \text{s}^{-1}$ . The cylindrical reactor vessel of internal size  $\sim 31$  mm (length) by 11 mm (diameter) was lined with Cd to reduce the  $(^{40}\text{Ar}/^{39}\text{Ar})_{\text{K}}$  correction factor, measured by analysis of  $\text{K}_2\text{SO}_4$  salt, to a low level<sup>25</sup>. Samples were placed in machined Al containers, with only one unknown in each reactor vessel in two separate containers of different size, sandwiched between those in which  $\text{K}_2\text{SO}_4$  was placed at either end of the vessel. Centrally located within each anorthoclase sample container was another cylindrical container in which the flux monitor, GA1550 biotite of K–Ar age 97.9 Myr (refs 23, 26), was placed. This geometrical arrangement of flux monitor and sample was developed<sup>27</sup> initially to overcome problems of large neutron flux gradients, but inversion of the reactor vessel at exactly halfway through the irradiation has virtually eliminated this difficulty, so that the neutron fluence can now be measured routinely to better than 1%.

Samples for  $^{40}\text{Ar}/^{39}\text{Ar}$  age spectrum measurements were heated by means of a radiofrequency generator for 45-min

intervals at successively higher temperatures in the Ar extraction system. Temperatures were measured by thermocouple and optical pyrometer; increments between successive steps are probably correct to  $\sim 10^\circ\text{C}$ , but the actual temperatures given in Table 1 are only approximate, because of relatively large temperature gradients over the Mo crucible in which the sample was heated. The gas from each extraction was purified and the Ar transferred directly to a Micromass 12 mass spectrometer, operated in the static mode and at an accelerating potential of 2 kV. Peak hopping, by varying the magnetic field, is used to focus the ion beams on the Faraday cup. Data are taken digitally on-line with a Hewlett–Packard 1000 computer, in which all data reduction is carried out. The amount of gas extracted at each step averaged  $\sim 2 \times 10^{-12}$  mol  $^{40}\text{Ar}$ , giving an ion beam of  $\sim 500$  mV using a  $10^{11}\text{-}\Omega$  resistor in the Cary 401 electrometer. Background in the mass spectrometer was measured routinely but normally was negligible.

Peak height data from the mass spectrometer were extrapolated to the time of admission of the gas into the machine using a least squares procedure and corrections applied for machine discrimination, decay of  $^{37}\text{Ar}$  and  $^{39}\text{Ar}$  and for neutron-induced interfering isotopes. Correction factors applied were:  $(^{36}\text{Ar}/^{37}\text{Ar})_{\text{Ca}} = 3.06 \times 10^{-4}$ ;  $(^{39}\text{Ar}/^{37}\text{Ar})_{\text{Ca}} = 7.27 \times 10^{-4}$  (ref. 25), and  $(^{40}\text{Ar}/^{39}\text{Ar})_{\text{K}} = 0.020 \pm 0.005$ , the average value measured on  $\text{K}_2\text{SO}_4$  during this work. For 78-1047 total fusion only, the value of the K correction factor was 0.066.

## Results

Data are given in Table 1 as amounts of each Ar isotope, derived from the measured sensitivity of the mass spectrometer. The relative amounts quoted for a given analysis are precise to within the uncertainties, but the absolute amounts may only be accurate to  $\sim 10\%$ . In a few cases almost all the  $^{37}\text{Ar}$  had decayed before analysis, but as the K/Ca ratio of these feldspars is large ( $> 50$ ), corrections for neutron-induced Ar from Ca are small to negligible. Data in Table 1 are not corrected for line blank, because the blank accounts for more than half the  $^{36}\text{Ar}$  in many analyses, and it is only known to  $\sim 50\%$ . Information is given in Table 1 so that blank corrected data can be calculated if desired.

Ages listed in Table 1 are calculated on the assumption that the non-radiogenic or trapped Ar in the samples has the composition of atmospheric Ar in which  $^{40}\text{Ar}/^{36}\text{Ar} = 295.5$ . These calculated ages are also shown as age spectra in Fig. 1.

In Fig. 2 data are shown for each step heating experiment on a correlation diagram with  $^{36}\text{Ar}/^{40}\text{Ar}$  as the ordinate and  $^{39}\text{Ar}/^{40}\text{Ar}$  as the abscissa<sup>28–30</sup>. If data from a step heating experiment define a good straight line on this diagram, it can be interpreted as a mixing line between non-radiogenic or trapped Ar, represented by a point on the ordinate, and Ar derived from K, represented by a point on the abscissa. Thus the isotopic composition of Ar for each gas fraction plots in Fig. 2 at a point determined by the proportion of the two end members. The intercept of the line with the ordinate defines the  $^{36}\text{Ar}/^{40}\text{Ar}$  ratio of the trapped component, and the intercept with the abscissa gives the  $^{39}\text{Ar}/^{40}\text{Ar}$  ratio, the reciprocal of which is proportional to age. This type of correlation diagram is preferred to the more common  $^{40}\text{Ar}/^{36}\text{Ar}$  versus  $^{39}\text{Ar}/^{36}\text{Ar}$  plot because problems associated with correlated errors are much less. Results of the regression analyses, using standard techniques<sup>31</sup>, are given in Table 2. In the regressions, the MSWD is a goodness of fit parameter; if this exceeds a value of  $\sim 2.5$  it can be concluded<sup>32</sup> that there is scatter of data points about the line significantly greater than can be accounted for by experimental error alone.

## Discussion

The  $^{40}\text{Ar}/^{39}\text{Ar}$  total fusion ages, measured on separate aliquots of the samples, agree well with one another, yielding a mean age of  $1.89 \pm 0.01$  Myr (Tables 1 and 2). In addition, the individual results are concordant with the K–Ar ages measured on the same samples (Table 2).



**Table 2** Summary of  $^{40}\text{Ar}/^{39}\text{Ar}$  ages derived from measurements on anorthoclase from pumice clasts, KBS Tuff, Koobi Fora Formation, Kenya

| Lab. no. | K-Ar age (Myr) $\pm 1$ s.d. | $^{40}\text{Ar}/^{39}\text{Ar}$ total fusion age (Myr) $\pm 1$ s.d. | Incremental total fusion age (Myr) $\pm 1$ s.d. | Data used in regression          | Plateau age (Myr) $\pm 1$ s.d. | $^{36}\text{Ar}/^{40}\text{Ar}$ versus $^{39}\text{Ar}/^{40}\text{Ar}$ regression |                                                | MSWD |
|----------|-----------------------------|---------------------------------------------------------------------|-------------------------------------------------|----------------------------------|--------------------------------|-----------------------------------------------------------------------------------|------------------------------------------------|------|
|          |                             |                                                                     |                                                 |                                  |                                | Age (Myr) $\pm 1$ s.d.                                                            | $(^{40}\text{Ar}/^{36}\text{Ar})_i \pm 1$ s.d. |      |
| 78-1038  | $1.89 \pm 0.02$             | $(1) 1.900 \pm 0.014$                                               | $1.890 \pm 0.026$                               | All data                         | $1.890 \pm 0.026$              | $1.860 \pm 0.011$                                                                 | $306.1 \pm 6.0$                                | 2.6  |
|          |                             |                                                                     |                                                 | Exclude 700°, 770° steps         | $1.881 \pm 0.023$              | $1.865 \pm 0.013$                                                                 | $302.6 \pm 6.8$                                | 2.7  |
|          |                             | (2) —                                                               | $1.880 \pm 0.025$                               | All data                         | $1.880 \pm 0.025$              | $1.853 \pm 0.010$                                                                 | $303.1 \pm 2.9$                                | 1.9  |
|          |                             |                                                                     |                                                 | Exclude 760° step                | $1.875 \pm 0.024$              | $1.874 \pm 0.013$                                                                 | $293.8 \pm 4.9$                                | 1.4  |
| 78-1047  | $1.88 \pm 0.02$             | $1.881 \pm 0.011$                                                   | $1.879 \pm 0.027$                               | All data                         | $1.879 \pm 0.027$              | $1.883 \pm 0.010$                                                                 | $296.0 \pm 2.0$                                | 1.9  |
| 79-14    | $1.88 \pm 0.02$             | $1.879 \pm 0.023$                                                   | $1.925 \pm 0.037$                               | All data                         | $1.925 \pm 0.037$              | $1.859 \pm 0.019$                                                                 | $304.1 \pm 5.0$                                | 10.3 |
|          |                             |                                                                     |                                                 | Exclude 600°, 760°, 1,250° steps | $1.893 \pm 0.034$              | $1.868 \pm 0.017$                                                                 | $289.3 \pm 5.0$                                | 6.4  |

$$\lambda = 5.543 \times 10^{-10} \text{ yr}^{-1}$$

$(^{40}\text{Ar}/^{36}\text{Ar})_i$  refers to composition of initial or trapped Ar.

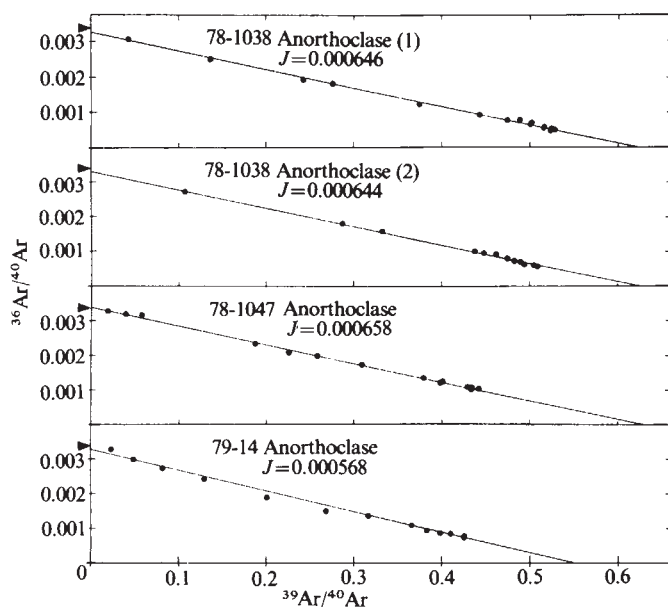
Incremental total fusion ages, calculated from all data obtained during a step heating experiment by combining the ages and errors according to the proportion of  $^{39}\text{Ar}$  in each step, agree satisfactorily with the directly measured total fusion ages, as the errors overlap in all cases (Tables 1 and 2).

Age spectra are shown in Fig. 1, in which the calculated age for each step, together with its error, is plotted against fraction of  $^{39}\text{Ar}$  released. The age spectra are similar to one another and are characterized by flat patterns or plateaus consisting of many steps which yielded essentially concordant ages over most of the release of  $^{39}\text{Ar}$ . Data are also shown on the  $^{36}\text{Ar}/^{40}\text{Ar}$  versus  $^{39}\text{Ar}/^{40}\text{Ar}$  plot in Fig. 2. Table 2 summarizes the results. Plateau and regression ages are derived using all data from each step heating experiment, as well as by excluding results from steps that give discordant ages. The criterion for exclusion of a datum was that the calculated age differed by more than twice its error ( $2\sigma$ ) from that of the plateau. Generally the steps excluded account for only a small proportion of the gas release at the beginning or end of the experiment, and the differences between the results are small (Table 2). Overall the plateau ages and those derived from the regressions form a remarkably concordant set ranging from 1.85 to 1.92 Myr (Table 2).

Two heating experiments were undertaken on anorthoclase 78-1038 to examine the reproducibility of an age spectrum. The two age spectra are similar (Fig. 1), each showing a well-developed plateau, the ages derived from which agree to within experimental error (Table 2). The second experiment yielded a virtually ideal plateau, comprising data from all steps except the first, which consisted of  $\sim 2\%$  of the total gas and which gave a high apparent age (Fig. 1). A similar effect is seen in the results from the first experiment, but the ages are only marginally high and are distributed over the first three gas fractions comprising  $\sim 5\%$  of the  $^{39}\text{Ar}$ . A possible explanation for the high ages for the early part of the release may be that some  $^{39}\text{Ar}$  was lost by recoil from the marginal parts of the crystal fragments<sup>33</sup>. Results from the second experiment indicate that the gas fraction extracted at 1,160 °C, but lost in the first experiment, is unlikely to have given an age very different from those obtained for adjacent steps.

Regression of data from both step heating experiments on sample 78-1038 gave ages systematically slightly younger than the plateau ages (Table 2). This is reflected also in the  $^{40}\text{Ar}/^{36}\text{Ar}$  values derived for the initial or trapped Ar, the values for which are higher than the atmospheric Ar value of 295.5. These differences, however, are small and not statistically significant, especially when calculated after exclusion of the few discordant results (Table 2). There is greater scatter of the data points about the regression line than can be accounted for by experimental error in the first experiment, but for the second experiment the MSWD value is  $<2.5$ , so that all scatter can be attributed to experimental error.

The two anorthoclase samples from pumice clasts in the KBS Tuff correlative in area 131 also yield age spectra exhibiting good plateaus (Fig. 1). Sample 78-1047 has a virtually ideal flat spectrum and all ages derived from the combined data are concordant at  $1.88 \pm 0.01$  Myr, irrespective of the manner by which they are calculated (Table 2). Gas from one step at 1,210 °C, comprising  $\sim 6\%$  of the total  $^{39}\text{Ar}$ , was lost in the experiment on 78-1047, but it is reasonable to assume that this gas fraction would have given results similar to those obtained from adjacent steps. The other anorthoclase from area 131, sample 79-14, yielded a plateau for more than 85% of the release (Fig. 1), but shows high apparent ages for the first 5% of  $^{39}\text{Ar}$  released and also for most of the last 8% of the release. The data points from this step heating experiment show significant scatter about the regression line, as indicated by the high value for the MSWD (Table 2). Even when the three most discordant points are excluded, there remains scatter in excess of that which can be accounted for by experimental error. If only those data are regressed that form the plateau between 5 and 92% of the  $^{39}\text{Ar}$  release, a line fitting to within experimental error is obtained (MSWD = 1.8). This regression yields an age of  $1.81 \pm 0.02$  Myr, and a  $^{40}\text{Ar}/^{36}\text{Ar}$  value for the trapped component of  $362 \pm 7$ , which is statistically significantly different from the value for atmospheric Ar. Such a result conflicts with data from the other samples, and may be quite accidental because all the



**Fig. 2** Correlation diagram showing Ar isotopic composition for each gas fraction of each step heating experiment on anorthoclase from pumice clasts in the KBS Tuff. A best fit straight line for each experiment is shown. Intercept on ordinate indicates composition of trapped Ar, and intercept on abscissa gives composition of the K-derived components, the inverse of which is proportional to age.  $\blacktriangleright$ , Isotopic composition of atmospheric Ar as indicated on ordinate.

points used in this regression plot are in a rather restricted portion of the correlation diagram with values for  $^{39}\text{Ar}/^{40}\text{Ar}$  between 0.31 and 0.43 (Fig. 2).

The flat  $^{40}\text{Ar}/^{39}\text{Ar}$  age spectra over much of the gas release for anorthoclase from pumices in the KBS Tuff suggest that the distribution of  $^{40}\text{Ar}^*$  in the crystals is relatively homogeneous. This conclusion would be correct if the effective dimension for  $^{40}\text{Ar}^*$  diffusion (see ref. 34) is less than the size of the crystal fragments used in the experiments. Some of the anorthoclase crystals exhibit closely spaced ( $\sim 2\ \mu\text{m}$ ) polysynthetic twins, so that it may be reasonable to propose that the distance between the twin planes is likely to be the effective dimension for  $^{40}\text{Ar}^*$  diffusion. Thus the lack of evidence for significant gradients of  $^{40}\text{Ar}^*$  in the feldspar concentrates analysed is interpreted as indicating that closed system behaviour has prevailed in the feldspar since crystallization, at least in respect of the K–Ar system.

Regression analyses of data from the three anorthoclase samples yield a mean age of  $1.87 \pm 0.01$  Myr, and suggest that the composition of the trapped or initial Ar in the samples is indistinguishable from atmospheric Ar (Table 2). The plateau ages agree at  $1.89 \pm 0.02$  Myr, and the directly measured  $^{40}\text{Ar}/^{39}\text{Ar}$  total fusion ages average  $1.89 \pm 0.01$  Myr. An age of  $1.88 \pm 0.02$  Myr is suggested as the best estimate from the  $^{40}\text{Ar}/^{39}\text{Ar}$  data. This is in excellent agreement with the mean conventional K–Ar age of  $1.89 \pm 0.02$  Myr derived from the results on seven anorthoclase samples analysed from pumices in the KBS Tuff in areas 105 and 131 (ref. 13). The concordancy of all these ages provides strong evidence that the anorthoclase has had a simple undisturbed history since crystallization from a rhyolitic melt. Geological evidence is interpreted<sup>3</sup> as indicating that deposition of the KBS Tuff occurred very soon after the explosive volcanism that produced the tuff and the pumice clasts, so that the measured ages are believed closely to approximate that of deposition. The simple geological history of the anorthoclase subsequent to its crystallization, incorporation in pumice clasts and deposition with the KBS Tuff, is consistent with the evidence that the tuff has never been covered by more than  $\sim 125$  m of sediment, and with the weakly indurated nature of the sediments in the sequence.

Finally I comment on the previously published<sup>11,16–19</sup>  $^{40}\text{Ar}/^{39}\text{Ar}$  results on anorthoclase from pumices in the KBS Tuff. The  $^{40}\text{Ar}/^{39}\text{Ar}$  total fusion ages measured on 10 different concentrates, as summarized by Fitch *et al.*<sup>19</sup>, range from 0.53 to 2.48 Myr, typically with quoted errors between 0.1 and 0.5 Myr. The proportion of  $^{40}\text{Ar}^*$  in these analyses generally is  $<20\%$  of the total  $^{40}\text{Ar}$  and commonly  $<10\%$  (ref. 18). On the basis of the large scatter in the ages and the small proportion of  $^{40}\text{Ar}^*$  in the gas extracted from the anorthoclase concentrates, I suggest that the results are analytically less precise than given by these authors.

The results of  $^{40}\text{Ar}/^{39}\text{Ar}$  age measurements by the step heat-

ing method on anorthoclase from KBS Tuff pumices, as reported by Fitch and coworkers<sup>11,16–19</sup>, are difficult to evaluate. This is partly because analytical data from only one of the eight experiments have been published. In two other cases a form of age spectrum diagram was given, and several correlation diagrams were shown, but interpretations only are provided for some of the step heating experiments. The authors state that each age spectrum is complex. Ages derived from these spectra range from  $1.10 \pm 0.32$  to  $2.48 \pm 0.01$  Myr (refs 11, 18, 19). They interpret ages of  $\sim 2.48$  Myr as reflecting crystallization of the anorthoclase, and the various younger apparent ages are attributed to subsequent thermal overprinting or disturbance of the feldspars. As noted previously<sup>13</sup>, geological evidence for such thermal events is lacking. I suggest that unrecognized analytical difficulties and larger than quoted errors must be invoked to explain these earlier  $^{40}\text{Ar}/^{39}\text{Ar}$  results.

## Conclusions

The  $^{40}\text{Ar}/^{39}\text{Ar}$  age spectra on the three anorthoclase separates from pumice clasts in the KBS Tuff show well-developed plateaus over most of the gas release. This is interpreted as indicating absence of significant  $^{40}\text{Ar}^*$  diffusion profiles in the anorthoclase concentrates, consistent with the geological evidence that the samples are unlikely to have been thermally disturbed since crystallization. The  $^{40}\text{Ar}/^{39}\text{Ar}$  total fusion ages, the plateau ages and those derived from the regressions are all concordant at  $1.88 \pm 0.02$  Myr, in excellent agreement with our<sup>13</sup> earlier conventional K–Ar age measurements. As deposition of the tuff is considered to have occurred soon after explosive eruption of the volcanic material, this age provides a good estimate for the KBS Tuff, which must now be regarded as extremely well dated. Such an age is late Pliocene, as the Pliocene–Pleistocene boundary has an estimated age of 1.7 Myr (ref. 35). The precise, accurate dating of the KBS Tuff, a marker bed in the sedimentary sequence exposed adjacent to and east of Lake Turkana, also provides a similarly precise age for tuff bed H2 in the sequence in the Omo Valley, just north of Lake Turkana, as geochemical data<sup>20</sup> indicate that the two tuffs are likely to be equivalent.

Finally, our work confirms that the  $^{40}\text{Ar}/^{39}\text{Ar}$  age spectrum method can be used successfully on Pliocene to Pleistocene rocks.

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- Behrensmeier, A. K. *Nature* **226**, 225–226 (1970).
- Vondra, C. F. & Bowen, B. E. in *Geological Background to Fossil Man* (ed. Bishop, W. W.) 395–414 (Scottish Academic, Edinburgh, 1978).
- Findlater, I. C. in *Koobi Fora Research Project Vol. 1* (eds Leakey, M. G. & Leakey, R. E. F.) 14–31 (Clarendon, Oxford, 1978).
- Leakey, R. E. F. *Nature* **226**, 223–224 (1970).
- Leakey, M. D. *Nature* **226**, 228–230 (1970).
- Isaac, G. L., Harris, J. W. K. & Crader, D. in *Earliest Man and Environments in the Lake Rudolf Basin* (eds Coppins, Y., Howell, F. C., Isaac, G. L. & Leakey, R. E. F.) 533–551 (University of Chicago Press, 1976).
- Coppins, Y., Howell, F. C., Isaac, G. L. & Leakey, R. E. F. (eds) *Earliest Man and Environments in the Lake Rudolf Basin* (University of Chicago Press, 1976).
- Bishop, W. W. (ed.) *Geological Background to Fossil Man* (Scottish Academic, Edinburgh, 1978).
- Leakey, M. G. & Leakey, R. E. F. (eds) *Koobi Fora Research Project Vol. 1* (Clarendon, Oxford, 1978).
- Cronin, J. E., Boaz, N. T., Stringer, C. B., & Rak, Y. *Nature* **292**, 113–122 (1981).
- Fitch, F. J., Hooker, P. J. & Miller, J. A. *Nature* **263**, 740–744 (1976).
- Curtis, G. H., Drake, R., Cerling, T. & Hampel, J. *Nature* **258**, 395–398 (1975).
- McDougall, I., Maier, R., Sutherland-Hawkes, P. & Gleadow, A. J. W. *Nature* **284**, 230–234 (1980).
- Gleadow, A. J. W. *Nature* **284**, 225–230 (1980).
- Drake, R. E., Curtis, G. H., Cerling, T. E., Cerling, B. W. & Hampel, J. *Nature* **283**, 368–372 (1980).
- Fitch, F. J. & Miller, J. A. *Nature* **226**, 226–228 (1970).

- Fitch, F. J., Findlater, I. C., Watkins, R. T. & Miller, J. A. *Nature* **251**, 213–215 (1974).
- Fitch, F. J. & Miller, J. A. in *Earliest Man and Environments in the Lake Rudolf Basin* (eds Coppins, Y., Howell, F. C., Isaac, G. L. & Leakey, R. E. F.) 123–147 (University of Chicago Press, 1976).
- Fitch, F. J., Hurford, A. J., Hooker, P. J. & Miller, J. A. *U.S. geol. Surv. Open File Rep.* **78-701**, 114–117 (1978).
- Cerling, T. E., Brown, F. H., Cerling, B. W., Curtis, G. H. & Drake, R. E. *Nature* **279**, 118–121 (1979).
- Merrill, C. M. & Turner, G. *J. geophys. Res.* **71**, 2852–2857 (1966).
- Turner, G. in *Origin and Distribution of the Elements* (ed. Ahrens, L. H.) 387–398 (Pergamon, Oxford, 1968).
- McDougall, I. & Roksandic, Z. *J. geol. Soc. Austr.* **21**, 81–89 (1974).
- Wang, S., McDougall, I., Tetley, N. & Harrison, T. M. *Earth planet. Sci. Lett.* **49**, 117–131 (1980).
- Tetley, N., McDougall, I. & Heydegger, H. R. *J. geophys. Res.* **85**, 7201–7205 (1980).
- Steiger, R. H. & Jäger, E. *Earth planet. Sci. Lett.* **36**, 359–362 (1977).
- Tetley, N. W. thesis, Australian National Univ. (1977).
- Turner, G. *Earth planet. Sci. Lett.* **11**, 169–191 (1971).
- Roddick, J. C., Cliff, R. A. & Rex, D. C. *Earth planet. Sci. Lett.* **48**, 185–208 (1980).
- Radicati di Brozolo, F., Huneke, J. C., Papanastassiou, D. A. & Wasserburg, G. J. *Earth planet. Sci. Lett.* **53**, 445–456 (1981).
- York, D. *Earth planet. Sci. Lett.* **5**, 320–324 (1969).
- Roddick, J. C. *Earth planet. Sci. Lett.* **41**, 233–244 (1978).
- Turner, G. & Cadogan, P. H. *Proc. 5th Lunar Sci. Conf. Geochim. cosmochim. Acta Suppl.* **5**, 1601–1615 (1974).
- Foland, K. A. *Geochim. cosmochim. Acta* **38**, 151–166 (1974).
- Berggren, W. A. *et al. Quat. Res.* **13**, 277–302 (1980).



# Early archaeological sites, hominid remains and traces of fire from Chesowanja, Kenya

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*Recent investigations of Lower Pleistocene sites at Chesowanja have yielded in situ Oldowan and Oldowan-like stone artefacts, evidence of fire and a fragmentary 'robust' australopithecine cranium. Burnt clay found at one artefact locality dated to  $>1.42 \pm 0.07$  Myr is the earliest known evidence of fire associated with a hominid occupation site.*

THE promise of the Pleistocene localities at Chesowanja, near Lake Baringo, was first shown in 1970, when a partial cranium of a 'robust' australopithecine was recovered<sup>1</sup>. Subsequent work by the late W. W. Bishop led to the recognition of early stone artefacts<sup>2-4</sup>, and systematic archaeological excavations began in 1978.

The exposed Lower Pleistocene sediments belong to the Chemoigut and Chesowanja Formations<sup>2,3</sup>. The Chesowanja basalt, dated to  $1.42 \pm 0.07$  Myr by K-Ar isotope dating<sup>5</sup>, is an important stratigraphic marker which immediately overlies the Chemoigut Formation and lies beneath the palaeosol member of the Chesowanja Formation.

## Archaeological sites in the Chemoigut Formation

The largest and most informative excavation in the Chemoigut Formation, GnJi 1/6E, was in the northern area (Fig. 1 inset), only 15 m from the most recently discovered hominid site. It covered 40 m<sup>2</sup>, and nearly 1,000 artefacts were found *in situ*, together with numerous faunal remains, including some complete limb bones. Bovids, equids, hippopotamus and crocodile are all represented among the fossil bones recovered (a fuller report of the fauna will be published elsewhere).

The trench was cut in largely homogeneous compacted clayey silts. The sharply defined distribution of finds suggests that they accumulated in a silting-up channel, which can only otherwise be recognized by occasional grit lenses at its base. The finds have a limited vertical distribution (more than 50% occurring within 20 cm); there is no evidence of size sorting of either artefacts or bone and most of the finds occurred within the silts well above the gritty lenses, suggesting a low-energy environment of deposition. The fresh quality of the artefacts also suggests limited movement after discard. Alteration of the water level, perhaps due to seasonal factors, may have led to down-cutting, followed by infilling with almost identical sediments. This accords well with the view held by Bishop *et al.*<sup>2,3</sup> that the localities represent sedimentation at the margins of a saline lake with a fluctuating water level. A similar abundance of antelope and crocodile remains has been noted in some of the Olduvai Bed I sites which have also been interpreted in terms of a lake margin environment<sup>6,7</sup>. The range of fauna, particularly the presence of hippopotamus and crocodile, suggests periods when the water was quite fresh<sup>7</sup>. Remarkable features in GnJi 1/6E were the flecks and lumps of red mineral matter scattered among the artefacts and bones (Fig. 2). A smaller trench, GnJi 1/5, about 100 m further north, also produced artefacts associated with a scatter of bones.

Excavations were also made in area 2 (GnJi 2) of the Chemoigut Formation, where hominid fossils were found in 1970 and 1974. Artefact scatters occur on the surface, but only one

rewarding *in situ* occurrence, GnJi 2/8, was found. This horizon, which occurred only about 15 m from the hominid found in 1970, was close to the surface and could not be followed further laterally because of modern erosion gullies. Area 4 of the Chemoigut Formation contains at least one site (not yet investigated), but area 3 has yielded no finds.

## Sites in the Chesowanja Formation

The Chesowanja basalt is exposed to the east of the Chemoigut Formation. It plunges steeply to the east and is overlain by the palaeosol member of the Chesowanja Formation (Fig. 1, GnJi 10). Its upper surface slopes gently to the west and is sealed in by fine-grained bedded tuffs. These were mapped as belonging to the Karau Tuffs<sup>2</sup>, which outcrop extensively to the west of Chesowanja, but further work has shown that they are a separate local unit, and hence as yet undated (A. Hill, personal communication).

Small artefacts were widespread on the surface of area 10, especially near exposures of the tuffs. Two trenches (GnJi 10/4 and 10/5) revealed dense concentrations of artefacts in the top 50 cm of the Chesowanja Formation palaeosols, immediately underlying the tuffs.

In excavation GnJi 10/5, about 1,500 artefacts were found *in situ* in an area of 25 m<sup>2</sup>. Bone fragments included fish and crocodile remains.

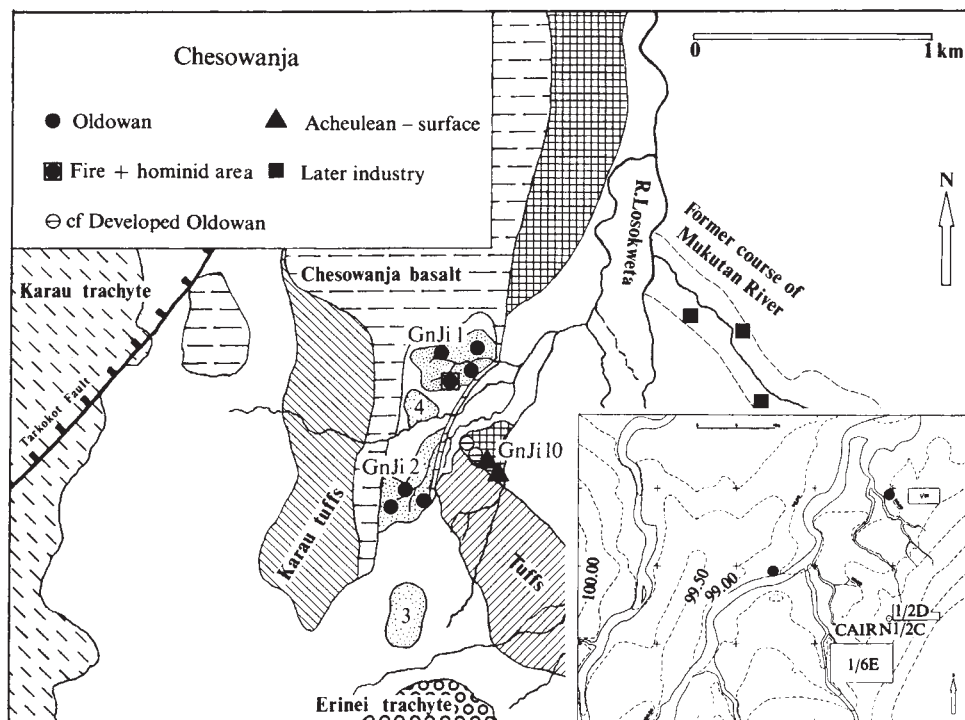
The density of finds and the thickness of the horizon, which included lenses of sand stratified in the clayey palaeosol, suggest that the hominids returned to the locality over a considerable period. Some of the artefacts are abraded, probably due to prolonged surface weathering, possibly from physical transport by water. One such abraded artefact had been retrimmed freshly on one surface and is strong evidence for recurrent use of a site.

In area 10, the artefacts originally discovered in 1973 included Acheulean bifaces<sup>1-3</sup>. However, bifaces are absent from the *in situ* assemblages excavated in 1978, but have been found on the surface immediately above the bedded tuffs. Trial trenches demonstrated that a later channel had cut through the bedded tuffs, down into the Chesowanja Formation palaeosols. Bifaces were found on the surface of the channel fill, where one small specimen was recovered *in situ*. Thus the bifaces are clearly of much later date and should not be considered components of the Chesowanja Formation industry.

## Affinities of the stone industries

The assemblages from the Chemoigut Formation can be assigned to the Oldowan industrial complex<sup>6</sup>, in the broadest sense. We previously assigned surface collections to the Developed Oldowan<sup>4</sup>, but the finely shaped discoids which prompted this designation may be explained partly by the especially tractable nature of the fine-grained lavas used in stone-working. Such specimens are not common *in situ* and the general character of the artefacts is compatible with material from Olduvai Bed I/Lower Bed II<sup>6</sup> (Fig. 3). Thus it is appropriate to designate this

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**Fig. 1** Locality map of Chesowanja (compare with ref. 3, Fig. 20:1). ▨, Chemoigut Formation exposures; ▩, Chesowanja Formation palaeosol. Later sediments are not distinguished on the map. In the inset: ●, hominid remains; —, modern gully edges; ----, contours at 50-cm intervals. Scale, 10 m between crosses.

as the Chemoigut Industry within the Oldowan complex. One crude biface, made from an elongated cobble, was isolated on the surface of the Chemoigut Formation, but this is not insufficient basis for postulating the existence of a contemporary Acheulean facies. Finished tools from the Chemoigut Formation are on average slightly larger than those from the Olduvai<sup>6</sup> or Karari<sup>8</sup> industries, but the assemblage from GnJi 2/8 does include small forms. Technologically, the artefacts are broadly comparable with those found on the Karari sites, but they do not include the high-backed heavy-duty scrapers characteristic of the Karari Industry.

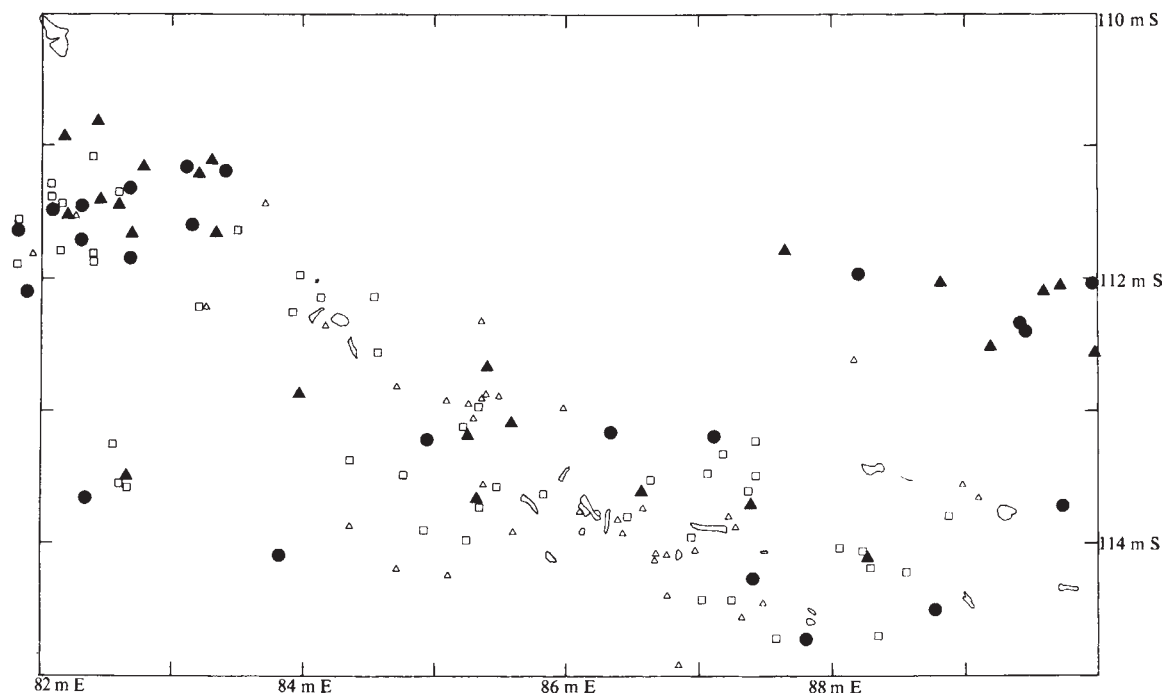
The industry from the Chesowanja Formation is also made mainly on lava, but some pieces are fashioned from welded tuff. The artefacts are strikingly small, and only 10 of more than 60 shaped tools weigh >50 g. The size of the artefacts was

apparently not constrained by the availability of raw materials, for lava must have been abundant locally. Due to its distinctive characteristics and stratigraphic position, we define this material as the Losokweta Industry (after a local river) and in purely descriptive terms regard it as 'cf. Developed Oldowan'.

The new stratigraphic observations suggest a much later date for the Acheulean artefacts<sup>3,4</sup>. They seem to resemble artefacts from the Kapthurin Formation<sup>9</sup> more closely than those from sites in the Baringo basin such as Kilombe<sup>10</sup>.

### New hominid evidence from Chesowanja

Fragments of a hominid cranium, KNM-CH 304, were discovered in two separate scatters, 15 m apart (Fig. 1), by Bernard Ngeneo and Wambua Mangao in 1978. The specimen consists of five cranial vault fragments labelled A-E. A is a fragment of



**Fig. 2** Finds in trench GnJi 1/6E (excluding flakes and flaking debris). ●, Heavy-duty tools; ▲, scrapers (light duty); △, bones; □, burnt clay. Axes are scaled in metres.



**Table 1** Artefact counts from Chesowanja determined by J. W. K. H. according to the typology used at Koobi Fora

|                                  | Chemoigut Industry |     | Losokweta Industry |
|----------------------------------|--------------------|-----|--------------------|
|                                  | 1/6E               | 2/8 | 10/5               |
| Area excavated (m <sup>2</sup> ) | 40                 | 16  | 25                 |
| Tools                            |                    |     |                    |
| Choppers                         | 9                  | 2   | 4                  |
| Polyhedrons                      | 8                  | 2   | 3                  |
| Discoids                         | 3                  | 4   | 19                 |
| Partial discoids                 | 5                  | —   | 2                  |
| Scrapers                         | 22                 | 1   | 29                 |
| Sundry                           | —                  | —   | 3                  |
| Total                            | 47                 | 9   | 60                 |
| Modified/edge damaged            | 28                 | 5   | 25                 |
| Debitage                         | 855                | 126 | 1,411              |
| Total artefacts                  | 930                | 140 | 1,496              |
| Unmodified cobbles/split cobbles | 13                 | 5   | 23                 |

occipital which includes lambda, inion and most of the left side of the occipital squame up to, and including, asterion; the surface bone is cracked and eroded. B is the left parietal including bregma, part of the coronal suture and a length of sagittal suture and parasagittal crest; C is the left temporal squame; D the right parietal including pterion and part of the squamosal suture; and E a small fragment of right parietal. Table 2 gives the length and thickness of A, B and relevant comparative material.

The most obvious diagnostic features of the cranium are ectocranial crests. The fused sagittal crest is 3 mm high at bregma, but 30 mm posteriorly it splits into two parasagittal crests which, at their highest point, project 11 mm from the vault surface. They continue onto the occipital bone where, about 20 mm posterior to lambda, they each turn abruptly laterally and fuse quite close to the midline with the nuchal crest to form a compound nuchal crest. The nuchal crest on the left side divides near to asterion and the inferior limb fuses with a separate crest running along the occipitotemporal suture. There is a small 'bare area' between inion and the point where the parasagittal crests diverge. Other features useful for taxonomic diagnosis are a series of coarse ridges and furrows which run anterolaterally from the sagittal suture in the direction of pterion; fine striae which mark the parietal bone just superior to the squamous suture and a pattern of venous sinus grooves on the endocranial surface of the occipital fragment, suggests that the main drainage pathway for venous blood was via an occipital venous sinus.

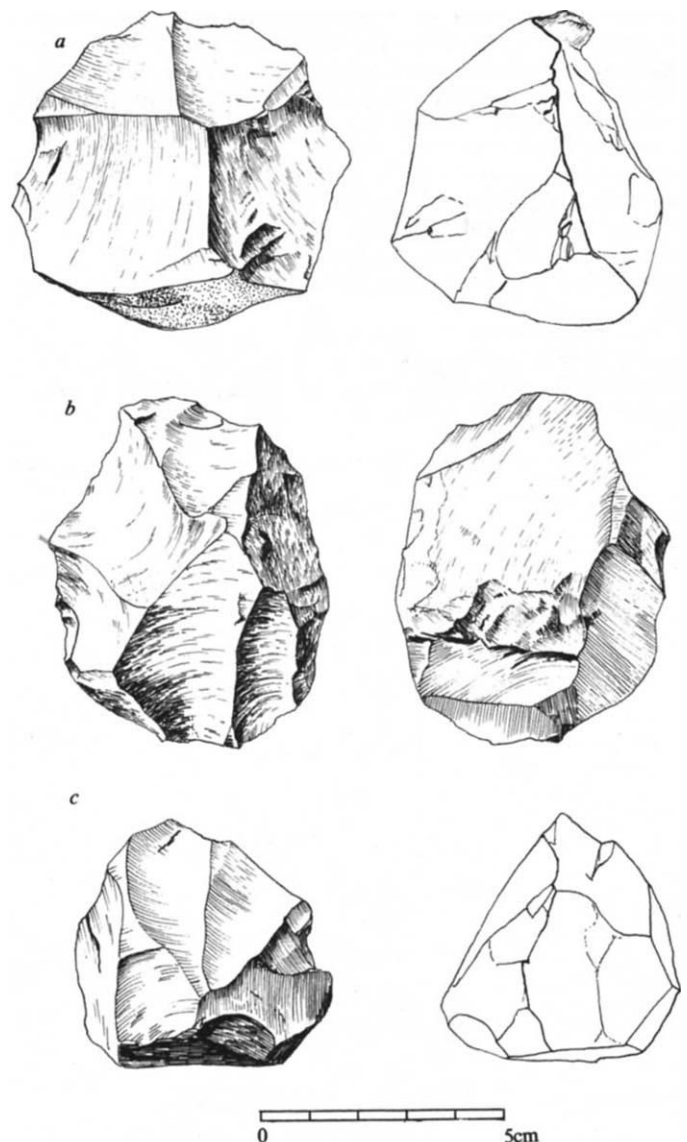
The combination of an anteriorly situated sagittal crest and a compound nuchal crest is shared with only one other fossil cranium, OH5, the type specimen of *Australopithecus boisei*<sup>11</sup> from Olduvai Gorge Tanzania. In another adult cranium belonging to the same taxon, KNM-ER 406, there is a substantial sagittal crest, but apparently no compound nuchal crest. In a juvenile cranium, L338y-6, from the Omo, the inferior temporal and superior nuchal lines nearly touch the left side whereas the inferior temporal lines are still 12 mm apart some 27 mm posterior to bregma<sup>12</sup>. Thus, if this individual had reached adulthood, a substantial compound nuchal crest would probably have been present. The adult skull KNM-ER 1805 from Koobi Fora, which still awaits formal attribution to a taxon, combines a compound nuchal crest with a sagittal crest, but in this case the sagittal crest is situated more posteriorly. It begins 30 mm posterior to bregma, and at stephanion the distance between the inferior temporal lines is at least 31 mm. The cranial vault morphology of fossils attributed to *Australopithecus africanus* suggests that sagittal crests may have occurred in some specimens, but there is no evidence that any of these crania had developed a compound nuchal crest.

The coarse ridges which mark the parietal are like those described by Tobias<sup>11</sup> in OH5, and similar features are also seen in KNM-ER 406. Finer striae—striae parietalis—are also

present on OH5, KNM-ER 406 and L338y-6. Rak<sup>13</sup> has carefully analysed these markings and he relates them to a special type of overlapping squamosal suture which has only been found in crania attributed to *A. boisei*. The pattern of venous sinus grooves, with a dominant occipital sinus, resembles that seen in OH5, SK 859 and SK 1585<sup>14</sup>, and a similar pattern is suggested by the preserved morphology in SK 46<sup>15</sup>. However, at least one East African 'robust' australopithecine specimen, L338y-6, has the more usual human pattern<sup>12</sup> (but Holloway<sup>16</sup> is cautious about attributing this specimen to *A. boisei*). Occipital sinus dominance is therefore best considered indicative, rather than diagnostic, of *A. boisei*.

Thus, although the new cranium is fragmentary, sufficient diagnostic areas are preserved to attribute it with some confidence to *A. boisei*, thereby confirming the presence of this hominid at Chesowanja.

This specimen fails to provide any new support for the proposal of Carney *et al.*<sup>1</sup> that the only other cranium known from Chesowanja, KNM-CH 1, represented an 'evolved' form of 'robust' australopithecine. Of the three possible features cited by these authors—a larger brain, a more flexed skull base and the reduction or refinement of masticatory apparatus—the new specimen is only complete enough to provide information about the last. The ectocranial crests suggests that the masticatory

**Fig. 3** Artefacts from the Chemoigut Formation. a, Chopper, b, discoid; c, polyhedron.

muscles in KNM-CH 304 were at least as well developed as they were in the two much better preserved 'robust' australopithecine crania from East Africa, KNM-ER 406 and OH5.

## Evidence and implications of fire

The possession of fire is a major component of cultural behaviour, and is universal in recent times even among hunters and gatherers. Evidence for fire is relatively common on later Acheulean sites<sup>17</sup> (~0.2–0.3 Myr), but rare in earlier sites; Vertesszöllos<sup>18</sup> (Hungary), Arago<sup>19</sup> (southern France) and Choukoutien<sup>17</sup> (China) are the main examples and none of these is believed to be older than 0.5 Myr.

If, however, fire had been used on earlier sites, evidence for it might have vanished. Isaac<sup>20</sup> noted that the fine charcoal remaining from wood fires is easily degraded in tropical conditions and readily removed by wind and water. Thus on open sites, baked material is likely to provide the best evidence of fire. Inconclusive evidence for fire has come from magnetic and thermoluminescence (TL) analysis of material from reddened areas of ground at site FxJj 20 East<sup>21</sup> on the Karari escarpment at Koobi Fora (~1.3–1.5 Myr) and from palaeomagnetic determinations on cobbles at an Acheulean site at Gadeb in Ethiopia<sup>22</sup>.

At Chesowanja, over 40 pieces of 'burnt clay' were found on the GnJi 1/6E site. They range in size from tiny flecks to lumps 5–7 cm across. They were thoroughly intermingled with the artefacts and bones (Fig. 2) and did not occur without this association. They could not have been introduced into the site after its formation. Samples of the supposed burnt material were

**Table 2** Measurements of KNM-CH 304 and relevant comparative material

|                           | KNM-CH 304 | OH5   | KNM-ER 406 | Sts 5 |
|---------------------------|------------|-------|------------|-------|
| <b>Parietal</b>           |            |       |            |       |
| Sagittal chord            | >75        | >76   | 80         | 76    |
| Thickness at bregma       | 5.3        | 5.5*  | —          | 4.0   |
| Thickness at asterion     | 2.5        | 6.8*  | —          | 7.0   |
| <b>Occipital</b>          |            |       |            |       |
| Inion–left asterion chord | 52         | 49    | 48         | 38    |
| Lambda–inion chord        | 37         | 36.2* | 45         | 39    |
| Thickness at lambda       | 10         | 9.0*  | —          | 7.0   |
| Thickness at asterion     | 14.5       | 13    | —          | 7.0   |

All measurements are in mm and, except those marked\* taken from ref. 11, were taken by B.A.W.

examined by one of us (D.W.). One sample possessed a magnetic moment of the order of  $10^{-4}$  A m<sup>2</sup> kg<sup>-1</sup>, which is broadly comparable with the magnetic moment of more recent samples of burnt clay<sup>23,24</sup>. On heating and cooling in zero magnetic field, the moment disappeared at 400 °C—a normal temperature for open camp fires, which seldom exceed 700 °C<sup>25</sup>.

Alternatively, the baked material could have resulted, not from a controlled camp fire, but from a 'wild' bush fire. However, although these create transient surface temperatures of up to 700 °C, the temperature then falls away rapidly below the surface<sup>26</sup>, so that baking of clay would be unusual. Investigations of baking around a recently burned tree stump near Chesowanja showed that the temperature was at least 200 °C higher than that of the fire at GnJi 1/6E<sup>21</sup>.

Thus, although the magnetic evidence is not in itself conclusive, it nonetheless strongly suggests that the Chesowanja clay was burned by a small, controlled fire. Future studies of the cooling rate may provide more specific evidence about its size. A possible explanation for the distribution of the detached lumps of burnt clay is provided by analogy with a later prehistoric hearth observed by one of us (J.A.J.G.) at Kilombe. A fire had baked the underlying sediments, which are now being removed by modern erosion. The burnt clay, being more resistant, was raised slightly above its surroundings, but was cracking and breaking up, and lumps of it were scattered for several metres downslope from the source.

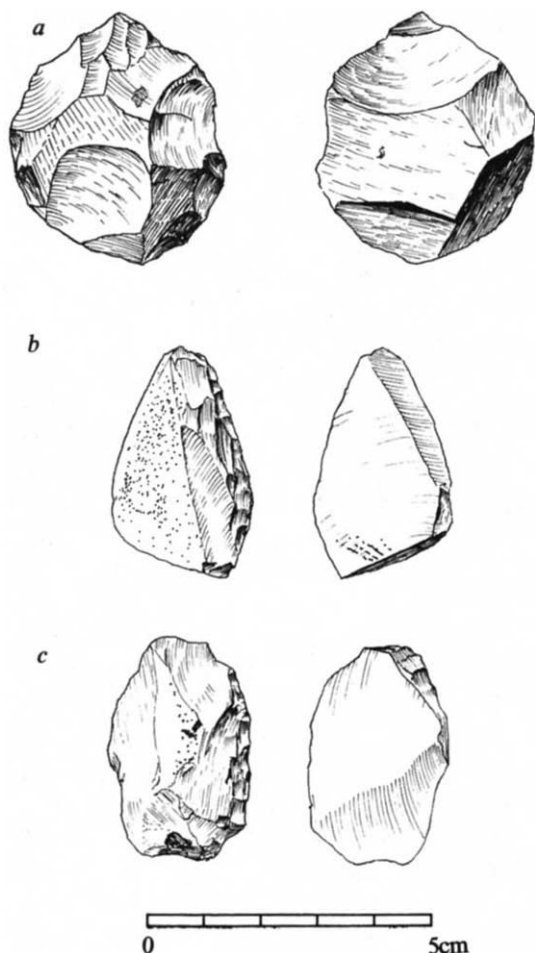
Although natural phenomena such as bush fires, lightning strikes and even volcanic heating could explain the burnt clay at Chesowanja, we are convinced, from examination of the whole occurrence *in situ*, that hominid activity is a much more likely explanation. Thus, the new find, together with the more tentative evidence from other sites, greatly strengthens the hypothesis that by 1.4 Myr hominids were using and controlling fire.

## Conclusions

At Chesowanja hominid remains of robust australopithecines have now been found close to archaeological finds at two separate localities almost 1 km apart. This association must be considered more than coincidental. At both sites non-hominid bones and bone fragments are closely associated with the artefacts, and it is likely that the artefact makers also consumed the meat.

The detailed dietary preferences of these robust australopithecines are not known, but preliminary evidence from studies of tooth microwear suggests that meat was not a significant component of their diet<sup>27</sup>. The Chemoigut Formation sites are approximately contemporary with early specimens of *Homo erectus* found elsewhere in Africa<sup>28</sup>. *H. erectus* was apparently responsible for the use of fire at Choukoutien and the use of fire by early representatives of the same hominid type at Chesowanja seems to us the most reasonable hypothesis. The principal alternative would involve the robust australopithecines being not merely meat-eaters, but also stone-tool makers and fire-users; we believe that the evidence is strongly against such a view.

If we accept the former hypothesis, then the presence of robust australopithecine fossils at both of these localities is



**Fig. 4** Artefacts from the Chesowanja Formation, site GnJi 10/5. *a*, Small discoid; *b*, *c*, scrapers.



difficult to explain on any basis except a chance association, or that their remains were brought to the site, along with those of other animals, by the makers of the artefacts.

The paucity of evidence bearing on these hypotheses underlines the necessity of locating and investigating new sites of the same period where there is well preserved and *in situ* evidence of hominid occupation.

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1. Carney, J., Hill, A., Miller, J. A. & Walker, A. *Nature* **230**, 509–514 (1971).
2. Bishop, W. W., Pickford, M. & Hill, A. *Nature* **258**, 204–208 (1975).
3. Bishop, W. W., Hill, A. & Pickford, M. in *Geological Background to Fossil Man* (ed. Bishop, W. W.) 309–327 (Scottish Academic, Edinburgh 1978).
4. Harris, J. W. K. & Gowlett, J. A. J. in *Proc. 8th Panafican Congr. of Prehist. & Quat. Stud.*, Nairobi (TILMIAF, 1980).
5. Hooker, P. J. & Miller, J. A. *Nature* **282**, 710–712 (1979).
6. Leakey, M. D. *Olduvai Gorge* Vol. 3 (Cambridge University Press, 1971).
7. Hay, R. L. *Geology of the Olduvai Gorge* (University of California Press, Berkeley, 1976).
8. Harris, J. W. K. & Herbich, I. in *Geological background to Fossil Man* (ed. Bishop, W. W.) 529–547 (Scottish Academic, Edinburgh, 1978).
9. Leakey, M., Tobias, P. V., Martyn, J. E. & Leakey, R. E. F. *Proc. prehist. Soc.* **35**, 48–76 (1969).
10. Gowlett, J. A. J. in *Geological Background to Fossil Man* (ed. Bishop, W. W.) 337–360 (Scottish Academic, Edinburgh, 1978).
11. Tobias, P. V. *Olduvai Gorge* Vol. 2 (Cambridge University Press, 1967).
12. Rak, Y. & Howell, F. C. *Am. J. phys. Anthrop.* **48**, 345–366 (1978).

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13. Rak, Y. *Am. J. phys. Anthrop.* **49**, 71–78 (1978).
14. Holloway, R. L. *Am. J. phys. Anthrop.* **37**, 173–186 (1972).
15. Tobias, P. V. in *Anthropology and Human Genetics* (Fischer, Stuttgart, 1968).
16. Holloway, R. L. *Am. J. phys. Anthrop.* **54**, 109–118 (1981).
17. Oakley, K. P. in *Social Life of Early Man* (ed. Washburn, S. L.) 176–193 (Wenner Gren, New York, 1961).
18. Kretzoi, M. & Vértess, L. *Curr. Anthropol.* **6**, 74–87 (1965).
19. De Lumley, H. (ed.) *La Préhistoire Française* Vol. 1, 823 (CNRS, Paris, 1976).
20. Isaac, G. L. I. *Ologesailie*, 94 (University of Chicago Press, 1977).
21. Barbetti, M., Wintle, A. & Flude, K. in Harris, J. W. K., thesis, Univ. California, 556–583 (1978).
22. Barbetti, M., Clark, J. D., Williams, F. M. & Williams, M. A. *J. Anthropologie* **18**(2,3), 299–304 (1980).
23. Walton, D. *Nature* **277**, 643–644 (1979).
24. Rogers, J., Fox, J. M. W. & Aitken, M. J. *Nature* **277**, 645–646 (1979).
25. Tylecote, R. F. *Metalurgy in Archaeology* (Arnold, London, 1962).
26. Stinson, K. J. & Wright, H. A. *J. Range Management* **22**, 169–174 (1966).
27. Walker, A. C. *Phil. Trans. R. Soc. B* **292**, 57–64 (1981).
28. Wood, B. A. in *Recent Advances in Primatology* (eds Chivers, D. J. & Joysey, J. A.) 349–372 (Academic, London, 1978).

## Structure of the pro $\alpha 2(I)$ collagen gene

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*Fifty-four kilobase pairs (kbp) of cloned chicken DNA containing the entire 38-kbp pro  $\alpha 2(I)$  collagen gene have been isolated and characterized. DNA sequence analysis of a select 4 kbp of the gene has precisely described 14 exons which comprise one-third of the sequences encoding the triple-helical domain of the collagen protein. These exons range in size from 45 to 108 base pairs (bp), are all multiples of the 9 bp that code for the repeating triplet, Gly-X-Y, and have an average size of 70 bp. About 50 introns interrupt this gene. Nevertheless, introns do not separate the coding sequences for the ends of the central triple-helical structural domain and the ends of the propeptide domains.*

THE collagens are a set of closely related proteins which provide the extracellular framework responsible for the structural integrity of nearly every organ and tissue in vertebrates. The correctly programmed spatial and temporal regulation of the production of collagen molecules is critical for growth and development. Therefore, the genes which encode these strategically important molecules deserve detailed investigation. Our investigations and those elsewhere have concentrated on the genes which encode the two chains that make up type I collagen, the most abundant of the collagen family.

The major part of the mature collagen consists of three polypeptide chains, called  $\alpha$ -chains, arranged in a long rod-like helical configuration. This region contains about 338 repeats of the amino acid triplet Gly-X-Y, where X and Y are often proline and hydroxyproline. Each polypeptide chain is synthesized as a propeptide with nonhelical regions at both the amino- and carboxy-terminal ends; the propeptides are removed by specific endopeptidases, leaving much shorter nonhelical regions, termed telopeptides, attached to both ends of the collagen helix. Five different types of collagen have been identified: they are made up from nine  $\alpha$ -chains which thereby require nine different genes<sup>1,2</sup>.

Recombinant DNA technology has made possible the isolation and structural analysis of several dozen unique eukaryotic genes. With the exception of the interferon genes, all unique genes in vertebrates have been found to contain noncoding

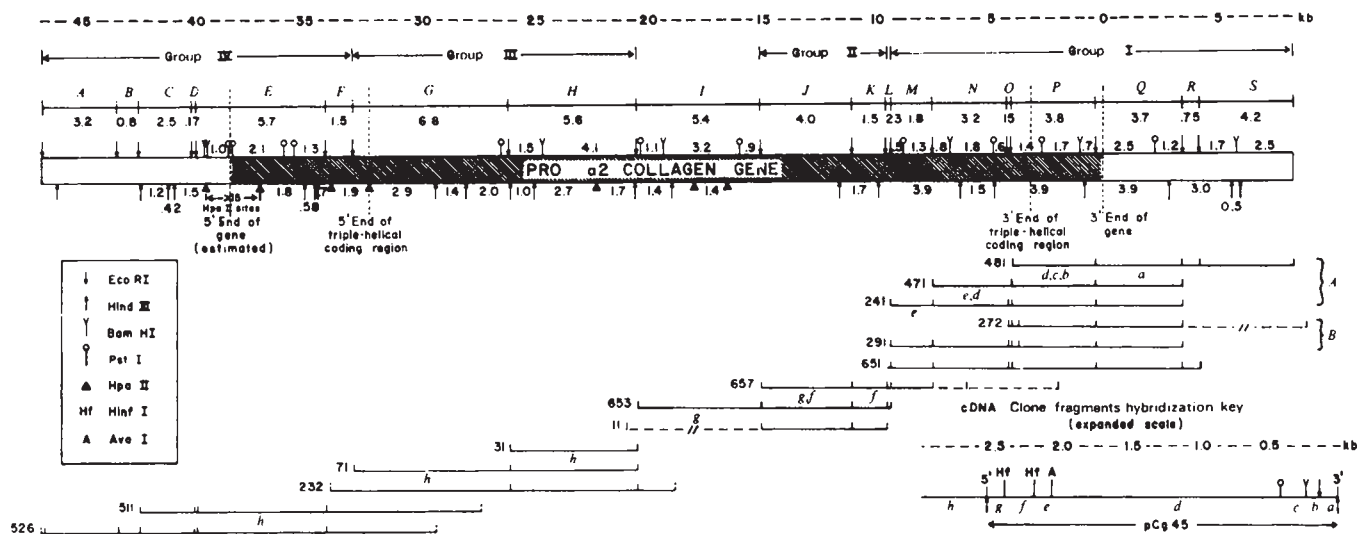
sequences (introns) which interrupt their coding sequences (exons). The number of introns ranges from a single intron, as in the rat insulin I gene<sup>3</sup>, to many introns, as in the *Xenopus laevis* vitellogenin gene which has 33 (ref. 4).

Our preliminary studies of the structure of the chicken pro  $\alpha 1(I)$  and pro  $\alpha 2(I)$  collagen genes, using actinomycin D/CsCl gradients and genomic DNA Southern blotting techniques, indicated that both these collagen genes would contain many introns<sup>5</sup>. This was dramatically confirmed for both the sheep and chicken pro  $\alpha 2$  collagen genes by electron microscopy studies of gene clones hybridized to procollagen mRNA. Almost 40 introns, ranging in size from <100 bp to >2,000 bp, could be clearly identified in the electron micrographs of gene clones spanning the entire chicken pro  $\alpha 2$  gene reported by Ohkubo *et al.*<sup>6</sup>. Seventeen introns were similarly identified in gene clones containing 60% of the sheep pro  $\alpha 2$  gene<sup>7</sup>. Thus, the pro  $\alpha 2$  collagen gene has emerged as the most highly interrupted gene so far examined.

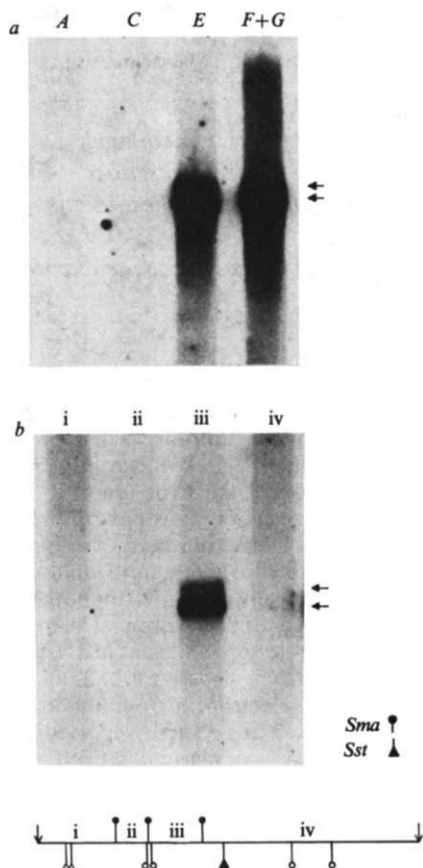
To investigate the intron-exon structure with precision, extensive sequence determinations are required. We have reported the DNA sequence analysis of seven exons in the helical region, ranging in size from 45 to 108 bp<sup>8</sup>. All are multiples of 9 bp coding for the repeating Gly-X-Y collagen triplet and each begins with the codon for glycine and ends with the codon for the Y residue. The exon sequences reported by Yamada *et al.*<sup>9</sup> have this same characteristic. However, the structure of the carboxy-terminal propeptide is quite different: we found the four exons there to be much larger, ranging in size from 189 to 444 bp<sup>8</sup>.

We report here the detailed structural analysis of 4 kbp of the collagen gene including the DNA sequence at both the amino-

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**Fig. 1** Restriction endonuclease map of pro  $\alpha 2$  collagen gene clones from chicken (*Gallus domesticus*). Clones containing *EcoRI* fragments I-S were isolated by hybridization to the cDNA clone pCg45 (ref. 25), those containing fragments G and H by cross-hybridization to pCg45 and those containing fragments A-F by hybridization to the *EcoRI*-*HpaII* fragment at the 5' end of *EcoRI* fragment G. As described previously<sup>8</sup>, the location and orientation of each of the clones were determined by hybridization of the clones digested with various restriction endonucleases to nick-translated fragments of pCg45 labelled a to g in the hybridization key. Fragment h refers to hybridization to kinased procollagen mRNA sequences not contained in pCg45. Vertical lines in the individual clones indicate *EcoRI* sites; dotted vertical lines indicate synthetic *EcoRI* sites generated by the linker molecules used in the construction of this  $\lambda$  library<sup>26</sup>.



**Fig. 2** Location of the 5' end of pro  $\alpha 2$  collagen gene. Calvaria mRNA was isolated, glyoxylated, electrophoresed on 1% agarose gels and blotted onto nitrocellulose as described by Thomas<sup>27</sup>. Isolated fragments were nick-translated and hybridized to lanes cut from the RNA blots. *a*, The *EcoRI* fragments used as probes are indicated above each lane (see Fig. 1 for locations of fragments). Arrows indicate positions of pro  $\alpha 2$  collagen mRNAs, located by hybridization to cDNA clone pCg45. The blot was overexposed to confirm lack of coding sequences in *EcoRI* A and C. The origin of the weaker, upper band is undefined, but may represent alternatively processed molecules or a stable precursor. *b* Shows hybridization to isolated subfragments of *EcoRI* E, the origins of which are shown below in the restriction endonuclease map of the fragment. Symbols as in Fig. 1. Hybridization to the *SmaI*-*EcoRI* subfragment iv is extremely weak, suggesting that there is little coding sequence in this fragment.

and carboxy-terminal junctions of the helical and propeptide coding regions of the gene, the DNA sequence of seven exons near the central portion of the gene which code for contiguous parts of the protein (residues 175-360) and the location, by DNA sequence analysis, of four other exons coding for residues 4-72.

### Isolation of DNA fragments

We have previously reported the isolation of recombinant  $\lambda$  clones containing ~80% of the pro  $\alpha 2$ (I) collagen gene, and have described in detail the structure of the 3' portion of this gene<sup>8</sup>. We have now isolated overlapping clones which can be arranged into a 54-kbp piece of contiguous genomic DNA containing the entire pro  $\alpha 2$  collagen gene. Figure 1 shows a partial restriction map of this DNA region, along with some of the  $\lambda$  clones which together span the gene. The cloning strategy is detailed in the Fig. 1 legend. The 5' end of the gene was located at the position indicated in Fig. 1 by two independent methods. Hybridization of labelled *EcoRI* fragments from the gene clones to procollagen mRNA indicated that the 5' end was probably located in fragment E, because positive hybridization was not obtained with any fragment lying 5' to this region (Fig. 2*a* and unpublished observations). Subdivision of fragment E with restriction enzymes and the use of these fragments to probe blots of procollagen mRNA enables us to locate the 5' end of the gene (Fig. 2*b*). These experiments do not rigorously exclude the possibility that there is another exon lying >8.3 kbp 5' to this region, nor that RNA transcription initiates at some point 5' and is so rapidly processed that it is not detected. However, the placement of the 5' end of the gene in this region is consistent with the results of *in vitro* RNA transcription using truncated gene clones as templates in the Manley<sup>10</sup> cell-free extract system (V.T., unpublished observations). This result, together with the hybridization experiments, place the 5' end of the pro  $\alpha 2$  collagen gene at the point indicated in Fig. 1. The 5 kbp of coding sequence for the pro  $\alpha 2$  collagen mRNA is thus spread over a total of 38 kbp of genomic DNA.

### Triple-helical coding sequences

Although electron microscope studies of mRNA-DNA hybrids may provide an overall picture of exon-intron arrangements in the pro  $\alpha 2$  collagen gene, only DNA sequence analysis can establish the exact size and coding capacity of the exons and the precise location of specific coding regions in the gene. As the



coding information of the gene is so dilute, it is necessary to locate DNA fragments which contain coding information in order to concentrate DNA sequence analysis where it is most valuable. Such regions were identified by hybridization to cloned cDNA or kinased procollagen mRNA and the locations determined more precisely by the use of restriction enzymes which cut sequences predicted to be abundant in the collagen triple-helical region but relatively infrequent elsewhere (see Fig. 4 legend).

The DNA sequences from the 5' end, the 3' end and central portions of the helical region of the pro  $\alpha 2$  collagen gene were then determined. Fifteen exons, including seven reported previously<sup>8</sup>, have been located (Fig. 3). The residue numbers of the amino acids in the triple-helical region coded for by each exon are given below. Figure 4 shows the DNA sequence of the genomic region containing the exons coding for residues 175–360 of the triple-helical region. Figure 4a displays the entire sequence of the 1.8-kbp *HpaII*–*EcoRI* fragment located at the 3' end of *EcoRI* fragment *H* (Fig. 1), while the sequencing scheme used is shown below. This fragment is seen to contain five exons which together encode residues 175–309. Figure 4b displays the DNA sequence of a region of *EcoRI* fragment *I* together with the sequencing scheme. Two exons are indicated, coding for residues 310–360. The seven exons contained within the sequences given in Fig. 4 thus code for contiguous parts of the protein (these exons are numbered 29–35 in Fig. 6.)

The DNA sequence determination of 14 complete exons coding for the triple-helical region has further detailed the remarkable structure of the pro  $\alpha 2$  collagen gene: it contains a very large number of exons, each with a correspondingly small amount of coding information; each exon is a multiple of the 9 bp which code for the repeating collagen triplet (Gly-X-Y). Of these 14 exons, 2 contain 45 bp, 7 54 bp, 3 99 bp and 2 108 bp. Although the most frequent exon size is 54 bp, it represents a total of only 7 out of the 14 exons. This contrasts with previous reports that all the exons in this gene contain 54 bp<sup>9</sup>.

The seven exons coding for residues 175–360 of the triple-helical region show an interesting alternation between large and small exons: 99-45-99-54-108-54-99. This pattern may be extended by including sequence determinations of two additional exons by Yamada *et al.*<sup>9</sup>. Exons coding for residues 379–411 (99 bp) and 412–429 (54 bp) were found; an exon of 54 bp, coding for 361–378, therefore almost certainly exists between these two exons and the seven exons whose sequence is given in Fig. 4. This would extend the alternating pattern to 10 exons, coding for 25% of the helical region. Such a pattern may well also exist in the 3' portion of the molecule, but it is clearly absent in the 5' end of the helical coding region of the gene. There is no obvious explanation for this alternating pattern nor why exons of other multiples of 9 bp are not found. Structural domains within the  $\alpha 2$  triple-helical region of sizes D (234 residues), D/6 (39 residues), D/11 (21 residues) and D/13 (18

residues) have been proposed on the basis of intra-chain amino acid sequence comparisons<sup>11</sup>. Although the latter domain size is the same as the most prevalent exon size, the fact that about half the exons encode 15, 33 or 36 residues indicates that there is little correlation between the exons and structural domains in the helical region.

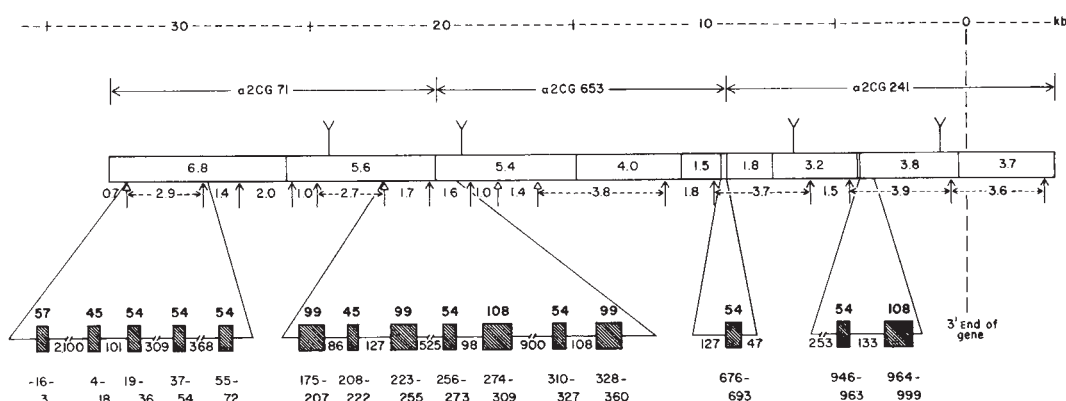
The finding that the triple-helical coding region of the pro  $\alpha 2$  collagen gene contains many small exons, each encoding a multiple of the Gly-X-Y repeat, suggests that the gene may have evolved by duplication. As seven of the eight exons first sequenced were reported to be 54 bp long, Yamada and co-workers proposed that the ancestral collagen gene was 54 bp and that during evolution this gene was duplicated to give rise to the present structure<sup>9</sup>. In this scheme, the large present-day collagen gene was formed by recombination between introns, followed by additions and deletions of 9 bp. The fact that only 7 of the 14 exons reported here are of 54 bp suggests a different mode of evolution for the collagen gene. If an ancestral triple-helical region gene of 54 bp is assumed, duplication by recombination between intergenic regions or introns (depending on the presence or absence of the propeptide coding regions) could indeed give rise to several 54 bp units. However, inasmuch as introns appear to be free to diverge rapidly, as compared with coding regions, recombination between different introns would be expected to be infrequent. Therefore, the repeating nature of collagen would make homologous recombination between different exons a far more likely event. We suggest that it is most likely that duplication took place in large part by recombination between exons (homologous unequal crossing over) rather than between introns. At each recombination point the exon size could change, readily accounting for exons differing from 54 bp. Recombination between two exons at non-identical positions within the 9 bp coding for the Gly-X-Y repeat would result in disruption of the helical structure of collagen, and thus be deleterious or fatal. Therefore, the exon sizes would always be maintained as a multiple of 9 bp.

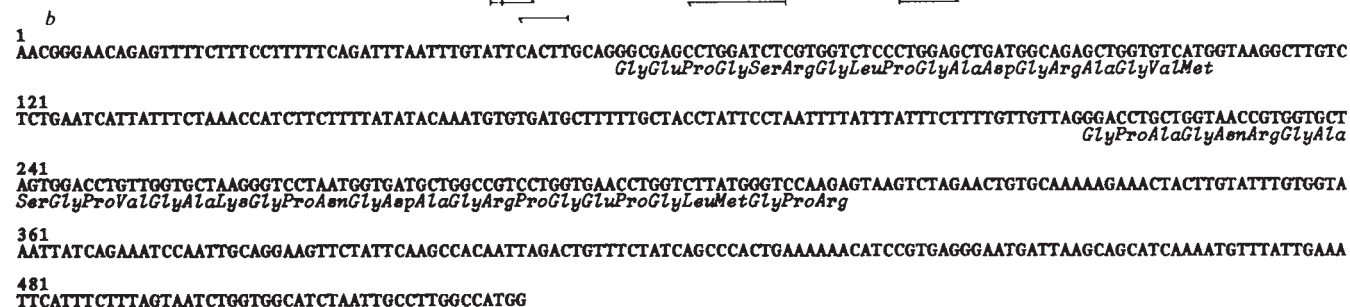
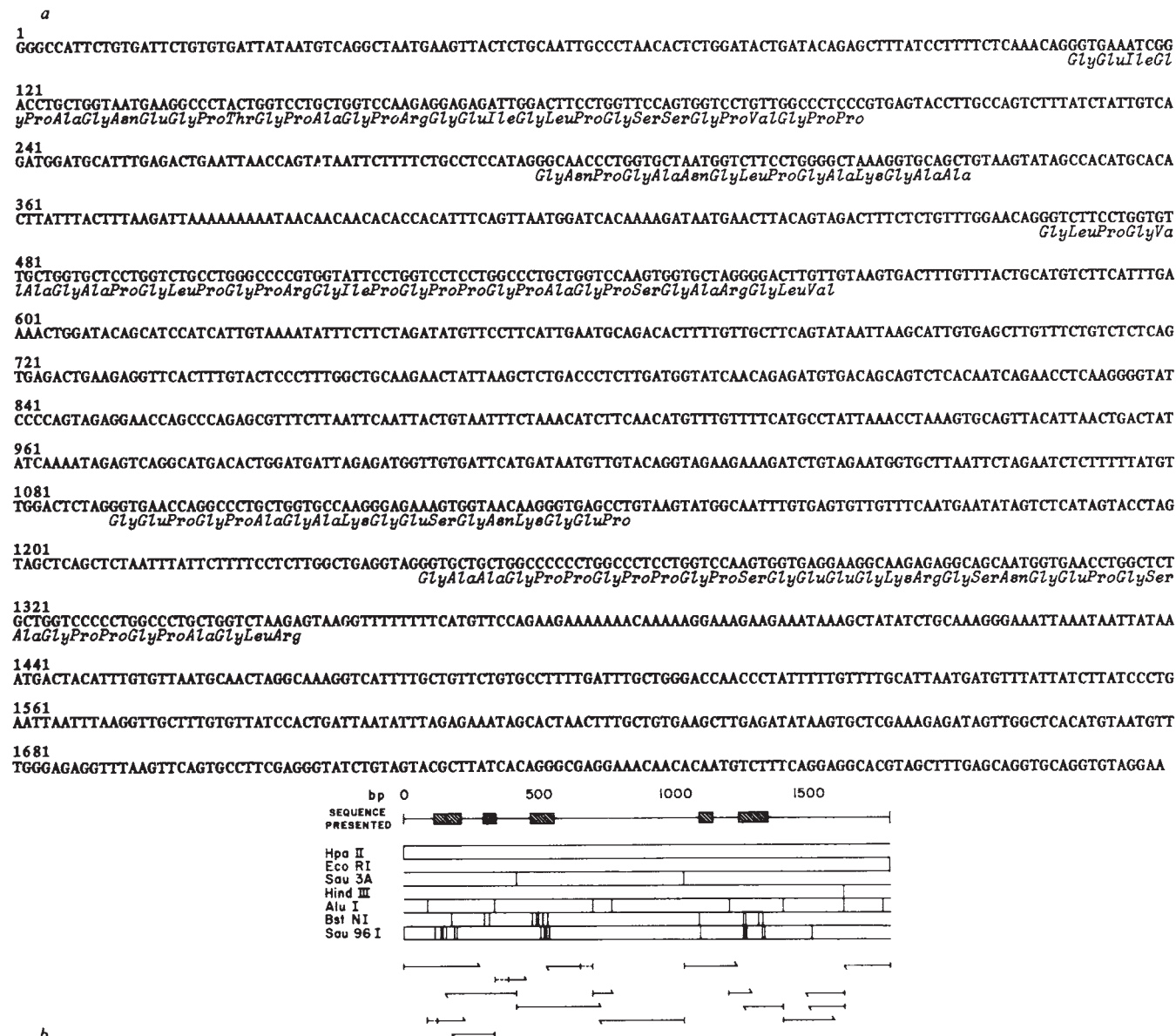
## Structural domain junctions

The DNA sequences of the exons coding for both the amino- and carboxy-terminal ends of the triple-helical region have also been determined. In both cases, these exons also code for the telopeptides and parts of the propeptides (Fig. 5). The exon at the amino-terminal end codes for the last 4 residues of the propeptide, all 12 residues of the telopeptide and the first 3 residues of the triple-helical region.

Although the amino acid sequence of the chicken amino-terminal propeptide has not been determined, this sequence is known for calf pro  $\alpha 1$  collagen<sup>12</sup>. The amino acid sequence around the amino-terminal propeptidase cleavage site for pro  $\alpha 1$  collagen is Asn-Phe-Ala-Pro↓Gln. The DNA sequence of the amino-terminal junction exon (Fig. 5) yields a similar

**Fig. 3** Location of sequences coding for the triple-helical region of the pro  $\alpha 2$  collagen gene by DNA sequence determinations. The coding regions (shaded boxes) are shown below the restriction map of the genomic DNA containing most of the pro  $\alpha 2$  collagen gene. Sizes of the exons and introns (in bp) are given above each. Below each exon are the residue numbers corresponding to the triple-helical region of collagen (numbering begins with 1 for the first glycine residue in the helical region). The 5'–most 57-bp exon codes for four residues of the amino-terminal propeptide, the telopeptide and the first three residues of the triple-helical region.





**Fig. 4** *a*, DNA sequence of the 1.8-kbp *HpaII*-*EcoRI* fragment at the 3' end of the *EcoRI* fragment *H* (coding for residues 175-309). *b*, DNA sequence of part of the 2.4-kbp *EcoRI*-*HpaII* fragment at the 5' end of the *EcoRI* fragment *I* (coding for residues 310-360). The sequencing schemes are shown below the DNA sequences. DNA sequences were determined by the method of Maxam and Gilbert<sup>28</sup>. Triple-helical coding sequences were located by restriction of isolated gene fragments with *BstNI* (an isoschizomer of *EcoRII*) and *Sau96I*. From the DNA sequences of the pro  $\alpha 2$  collagen triple-helical region determined from the cDNA clones, many of the Pro-Gly sequences of collagen will contain *BstNI* sites, CC(A/T)GG. *Sau96I* recognizes the sequence GGNCC, which is also predicted to be very common in the triple-helical coding regions as collagen has many Gly-Pro sequences. The *BstNI* and *Sau96I* sites identified by DNA sequence determinations are indicated on the last two lines of the restriction map in *a*. All 13 *BstNI* sites in this 1.8-kb fragment of DNA can be seen to fall within the collagen coding sequences (indicated by the shaded boxed above), while 16 of the 18 *Sau96I* sites also lie within coding regions. Thus, the correlation of these sites with triple-helical coding regions is excellent. The amino acid sequence for residues 175-327 of chicken  $\alpha 2$  collagen has not been determined; however, the calf collagen sequence is known<sup>29</sup>. Of the 153 residues determined by DNA sequence analysis here, 133 are identical to the calf sequence, indicating an 87% homology. Only one instance was noted where the DNA-derived amino acid sequence differs from that determined by amino acid sequencing of chicken  $\alpha 2$  collagen. The DNA sequence indicates that residues 339 and 341 are valine and alanine, whereas the reverse was found by amino acid sequencing<sup>30</sup>. Although it is possible that this represents an allelic difference, the discrepancy may be due to an error in the amino acid sequence determination, as the lack of a thermolysin site which should exist preceding the valine residue was noted when the protein sequence was presented.





1 ACCCTTCTCTACTGAATCAAGTCTTTCTGGAAACAGATGAATAGAAATGTATTGTAAAAAGGGTCATGTGTACTGAGTITTAAGTATGTTGCTTCAACATCTGTCTAGCAAATGAGGTAA  
121 TGGATACATGTAGAATGAGGGTTATCAGTGCTTTCTGAAGTAAGAATTGTAGTGACTTTTATTTCAAATTTTTGTTCATCTACAGAATTTTGTGCTCAGTATGATCCATCTAAAGCGGCT  
241 GACTTTGGCCCCGGACCTATGGTAAAGTATATGATTTAACACTTGGTAACTTGCATAAAACATGTATTTAAGTGTACTCCAG  
AspPheGluProGluProMet AsnPheAlaAlaGlnTyrAspProSerLysAlaAla

**C-terminal:**

1 AAGACTTCAGAAAGGACAAACGCATGTGTGGAAATAGTCAGCTGAAAGAATCGTATTTATAATAGAATACTAATTGTCTTTTGCTTTTGAAAACTTTAGGGCCCTCCTGGCCCTCTCTGGTC  
GlyProProGlyProProGlyP

121 CCCCTGGCCCCCTGGTCCCAATGGTGGCGGATAGAAAGTTGGCTTTGATGCAGAACTACTACGGGGCTGATCAGCCTTCTCTCAGACCCAAGGATTATGAAGTTGATGCCACTCTGAAAA  
roProGlyProProGlyProAsnGlyGlyGlyTyrGluValGlyPheAspAlaGluTyrTyrArgAlaAspGlnProSerLeuArgProLysAspTyrGluValAspAlaThrLeuLysT

241 CATTGAACAACCAAATTGAGACCTGCTGACCCAGAAAGGCTCCAAAAAGAACCGGGCTCGCACTGCGGTGACCTCAGACTTAGCCACCCAGAATGGAGCAGCGGTACGTGGTGCCAGA  
hrLeuAsnAsnGlnTleGluThrLeuLeuThrProGluGlySerLysLysAsnProAlaArgThrCysArgAspLeuArgLeuSerHisProGluTrpSerSerGly

361 TGTTTCCTCTTTCTGGCTCAGTAAGTCAITTTCA

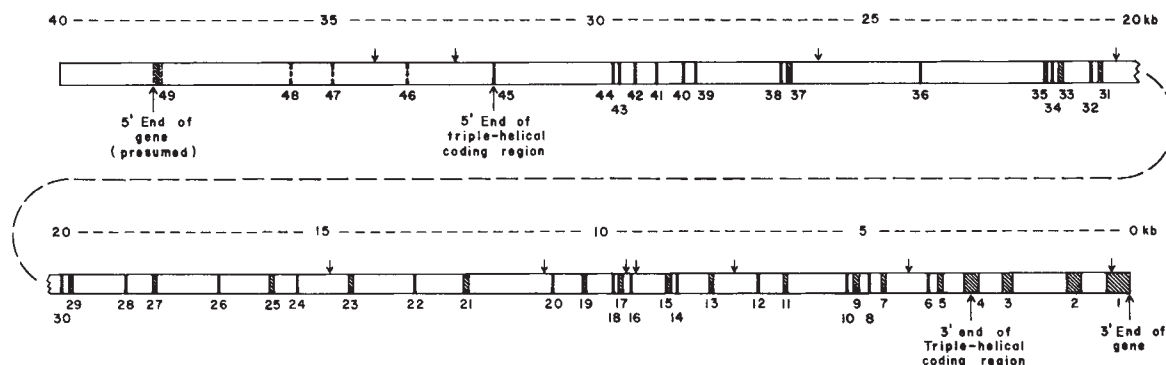
**Fig. 5** DNA sequences of the genomic regions containing the exons coding for the ends of the helical region. Propeptide coding regions are indicated by open boxes, telopeptide coding regions by solid boxes and triple-helical coding regions by shaded boxes. The amino-terminal junction exon codes for the last 4 residues of the propeptide, all 12 residues of the telopeptide and residues 1–3 of the helical region. The carboxy-terminal junction exon codes for residues 1,000–1,014 of the helical region, all 15 residues of the telopeptide and the first 53 residues of the propeptide. Splicing probably occurs within the last glycine codon. The 112-bp *Hpa*II fragment within the carboxy-terminal junction exon is present in both the gene clones and cDNA clones; its sequence was determined only from the cDNA clone<sup>31</sup>.

sequence for the presumptive propeptidase cleavage site: Asn-Phe-Ala-Ala↓Gln. As no homology can be found between the calf propeptide amino acid sequences located 5' to these in calf and the amino acid sequence which would result from the chick DNA sequence in this region, and as a CAG (the canonical splice junction sequence, see below) immediately precedes the codon for Asn, this exon presumably starts with the above conserved pentapeptide sequence. The following 14 residues, which include the first 3 residues in the triple-helical region, are identical with the amino acid sequence derived from chick skin  $\alpha 2$  collagen<sup>13</sup>. At the carboxy-terminal end<sup>8</sup>, the situation is similar except that larger portions of the propeptide and triple-helical coding regions are included in the exon coding for the telopeptide: a single exon codes for 15 residues of the helical region, all 15 residues of the telopeptide and the first 53 residues

of the propeptide. That these three *structural* domains (at both ends of the collagen molecule) are not separated by introns, as might have been expected, may possibly be explained in that they consist of a specific *functional* domain, namely the endopeptidase cleavage site.

### Intervening sequences

The 19 exons whose DNA sequences have been determined provide an equal number of intron-exon junction sequences (Table 1). In the triple-helical region, there is always an ambiguity as to the precise splice point due to the duplication of Gs at each end of the intron and there is a special problem in defining the exact splice points in the region which codes for the carboxy-terminal propeptide; three nucleotides at both junctions of exon



**Fig. 6** Location of coding sequences in the pro  $\alpha 2$  collagen gene. Forty kilobases of genomic DNA containing the pro  $\alpha 2$  collagen gene are represented by two bars, both oriented 5' to 3' (left to right). Exons are indicated by vertical bars, numbered 1 to 49 (3' to 5'). The four exons at the 5' end of the gene have been identified only by electron microscope visualization of RNA-DNA hybrids by Ohkubo *et al.*<sup>6</sup>, and are thus indicated by dotted lines. The exon sizes in the helical region whose DNA sequences have not been determined have been indicated such that the small-large alternating pattern extends from exon 38 to exon 5; this is consistent with electron microscope measurements. Arrows ( $\downarrow$ ) indicate *EcoRI* sites. Exons whose DNA sequences have been determined are as follows. In this article and ref. 8: exon numbers 45 (amino-terminal structural domain junction); 44-41 (residues 4-72); 35-29 (residues 175-360); 16 (residues 676-693); 6 and 5 (residues 946-999); 4 (carboxy-terminal structural domain junction); 3, 2 and part of 1 (carboxy-terminal propeptide). By other researchers<sup>9</sup>: 43-41 (residues 19-72); 27 and 26 (residues 379-429); 10 (residues 838-855); 9 (residues 856-891); 8 (residues 892-909). Only the ends of exon 9 have been sequenced, and therefore it has not been established whether this is one or two exons.

Table 1 Intron-exon junction sequences

| Residue nos | Intron  | Exon                    | Intron    |                               |
|-------------|---------|-------------------------|-----------|-------------------------------|
| 1           | -3      | TCATCTACAG              | AAT---ATG | (junction)                    |
|             |         | asn met                 |           |                               |
| 2           | 4-18    | TCTTCCCTAG              | GGT---CCT | (triple-helical)              |
|             |         | gly pro                 |           |                               |
| 3           | 19-36   | TCTTGTCTAG              | GGT---ACA | GTAAGTACAG                    |
|             |         | gly thr                 |           |                               |
| 4           | 37-54   | TATTTGACAG              | GGT---GAT | GTAAGTCATT                    |
|             |         | gly asp                 |           |                               |
| 5           | 55-72   | CATTTTTCAG              | GGT---CAA | GTAAGTATGT                    |
|             |         | gly gln                 |           |                               |
| 6           | 175-207 | TCTCAAACAG              | GGT---CCC | GTGAGTACCT                    |
|             |         | gly pro                 |           |                               |
| 7           | 208-222 | GCCTCCATAG              | GGC---GCT | GTAAGTATAG                    |
|             |         | gly ala                 |           |                               |
| 8           | 223-255 | TTTGGAACAG              | GGT---GTT | GTAAGTGACT                    |
|             |         | gly val                 |           |                               |
| 9           | 256-273 | TGGACTCTAG              | GGT---CCT | GTAAGTATGG                    |
|             |         | gly pro                 |           |                               |
| 10          | 274-309 | GCTGAGGTAG              | GGT---AGA | GTAAGGTTTT                    |
|             |         | gly arg                 |           |                               |
| 11          | 310-327 | TCACTTGCAG              | GGC---ATG | GTAAGGCTTG                    |
|             |         | gly met                 |           |                               |
| 12          | 328-360 | TTGTTGTTAG              | GGA---AGA | GTAAGTCTAG                    |
|             |         | gly arg                 |           |                               |
| 13          | 676-693 | TCACACATAG              | GGT---CCT | GTAAGTCCTC                    |
|             |         | gly pro                 |           |                               |
| 14          | 946-963 | TTCCCAATAG              | GGC---AGG | GTATGTGAAT                    |
|             |         | gly arg                 |           |                               |
| 15          | 964-999 | TTTCTTCTAG              | GGT---GCT | GTGAGTATAA                    |
|             |         | gly ala                 |           |                               |
| 16          | 1,000-  | AAAACTTTAG              | GGC---GCG | GTACGTGGTG                    |
|             |         | gly g                   |           | (junction)                    |
| 17          |         | TTCCTCAAAG              | GGT---CAG | GTATGTGATG                    |
|             |         | ly gln                  |           | (carboxy-terminal propeptide) |
| 18          |         | TCTTTTTCAG <sup>g</sup> | TTT---TCT | GTAAGTAACA                    |
|             |         | phe ser                 |           |                               |
| 19          |         | TCTCTTGCAG              | AAA---    | ND                            |
|             |         | lys                     |           |                               |

ND, not determined.

17 are duplicated at the adjacent exon junction, so that four splice points are possible. The splice points in Table 1 have been aligned so that they conform with the GT---AG rule<sup>14</sup>; this requires splicing within the glycine codon at the ends of exons 16/17. With the sequences aligned in this manner, 11 introns begin with GTAAGT, and 7 others differ from this by a single base change. Eighteen introns end with (C/T)AG. These sequences agree well with those found for other eukaryotic genes and are indeed homologous to the 5' part of the U1 RNA which has been proposed to be involved in the splicing process<sup>15,16</sup>. Because every exon in the triple-helical region starts with a glycine residue, the first nucleotide in each is a G, as is usually found in other eukaryotic genes. However, the G which is commonly found at the 3' end of the exons in most genes occurs infrequently in the pro  $\alpha 2$  collagen gene. There appears to be a strong bias against the use of G in the third position of codons in this gene.

The introns defined by DNA sequence analysis range in size from 86 bp to more than 2,000 bp. The average G + C content of the introns is about 35%, in striking contrast to the G + C-rich (62%) coding sequences, resulting in an average G + C content of 40%. The structure of the pro  $\alpha 2$  collagen gene therefore consists of many small G + C-rich coding regions embedded in A + T-rich stretches of DNA.

There seems to be little homology between different introns. Computer-generated homology plots<sup>17</sup> show that the longest homologies are ~10 bp. However, some of the introns seem to contain DNA sequences which are repeated in other parts of the genome. *EcoRI* fragments *G*, *H* and *I* all hybridize to a large size range of DNA fragments on Southern blots of chicken DNA (J.W., unpublished data). The observation that some of the introns contain sequences which are repeated in distal parts of the genome is similar to that found for the chick X gene, which lies near the ovalbumin gene<sup>18</sup>, and the conalbumin gene<sup>19</sup>, but contrasts with the structure of the ovalbumin and globin genes, where the introns contain only unique DNA<sup>26,21</sup>.

## Number of exons

The DNA sequence determinations of various regions of the chicken pro  $\alpha 2$  collagen gene indicate that the structure of this gene is complex. By integrating the data from the various techniques used to characterize this gene, we deduce the detailed picture of the gene's structure presented in Fig. 6. The placement and sizes of the exons, indicated by vertical bars in the figure, have been determined by DNA sequence analysis, Southern blot restriction mapping and electron microscopy studies of genomic DNA hybridized to procollagen mRNA in R-loop conditions. The parts of pro  $\alpha 2$  collagen for which individual exons code are detailed in Fig. 6 legend. Certain details differ from the arrangement of exons presented by Yamada *et al.*<sup>9</sup> and Ohkubo *et al.*<sup>6</sup>, deduced from electron microscopy studies and limited DNA sequence analysis. The DNA sequence analysis of multiple exons coding for contiguous parts of the protein ensure that the number of exons in these regions and their placement with respect to the map presented in Fig. 6 are correct.

We have indicated in Fig. 6 that the pro  $\alpha 2$  collagen gene contains 49 exons. This is only an approximate number, as some of the exons whose DNA sequences have not been determined are identified only by electron microscopy, in which small exons or introns may be undetected. However, the figure is a fairly accurate representation of the location and minimal number of exons. Note that the density of exons in the 3' half of the gene is higher than that in the 5' half; there is more than twice as much coding information in the last 19 kbp of the gene as in the first 19 kbp. From the 14 triple-helical coding exons whose sequences were determined, the average exon size is ~70 bp. This predicts a total of 41 to 42 exons for the triple-helical coding region (excluding the junction exons). Adding to this the four exons coding for the carboxy-terminal propeptide and several for the amino-terminal propeptide, we predict a total of about 50 exons in the pro  $\alpha 2$  collagen gene.



The highly interrupted nature of the pro  $\alpha 2$  collagen gene requires the transcription of 38 kbp of DNA and the precise removal of about 50 introns to produce a single mature 5-kbp messenger RNA. The large number of splicing reactions which must take place certainly suggests that RNA splicing is in some sense processive, that is, that the enzyme would start at an intron/exon junction and search progressively down the intron DNA for a complementary splice point<sup>22</sup>. An alternative random mechanism would probably result in the excision of one or more coding regions, producing an error frequency intolerable for a gene with 50 exons.

Regardless of whether the introns arose concurrently with the evolution of the gene or were inserted after the gene was developed, it seems clear that there must be a reason for maintenance for this highly interrupted structure. It is possible that the introns help to stabilize the coding sequences. Investigation of the silk fibroin gene, which also has highly internally repetitive coding sequences but only one intron in the entire gene, has shown that different alleles of the gene give rise to differently sized fibroin molecules<sup>23</sup>. The size differences appear to result from changes in the size of the repetitive parts of the genes, presumably a result of recombination between these parts in misaligned genes, that is, homologous unequal crossing-over. By embedding the repetitive collagen coding sequences in nonhomologous DNA (the introns), the probability of homologous recombination would be reduced<sup>24</sup>, thus stabilizing the size and structure of the collagen molecule.

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1. Bornstein, P. & Sage, H. A. *Rev. Biochem.* **49**, 957–1003 (1980).
2. Eyre, D. R. *Science* **207**, 1315–1322 (1980).
3. Lomedico, P. *et al. Cell* **18**, 545–558 (1979).
4. Wahli, W., Dawid, I. B., Wyler, T., Weber, R. & Ryffel, G. U. *Cell* **20**, 107–117 (1980).
5. Wozney, J. M., Hanahan, D. A., Fuller, F. H. & Boedtker, H. *Fedn Proc.* **38**, 617 (1979).
6. Ohkubo, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7059–7063 (1980).
7. Shaffer, M., Boyd, C., Tolstoshev, P. & Crystal, R. *Nucleic Acids Res.* **8**, 2241–2253 (1980).
8. Wozney, J., Hanahan, D., Morimoto, R., Boedtker, H. & Doty, P. *Proc. natn. Acad. Sci. U.S.A.* **78**, 712–716 (1981).
9. Yamada, Y. *Cell* **22**, 887–892 (1980).
10. Manley, J. L., Fire, A., Cano, A., Sharp, P. A. & Gelfand, M. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3855–3859 (1980).
11. Hofmann, H., Fietzek, P. P. & Kuhn, K. J. *molec. Biol.* **141**, 293–314 (1980).
12. Olsen, B. R. & Berg, R. A. in *Secretory Mechanisms* (eds Hopkins, C. R. & Duncan, D. J.) (57–78) (Cambridge University Press, 1979).
13. Kang, A. H. & Gross, J. *Biochemistry* **9**, 796–804 (1970).
14. Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. & Chambon, P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4853–4857 (1978).

## Conclusion

The isolation of clones spanning 54 kbp of genomic DNA and DNA sequence determinations of over 4 kbp of this region, have shown that the chicken pro  $\alpha 2(I)$  collagen gene has a bizarre structure. The 5 kbp of coding sequences are spread out over about 38 kbp of genomic DNA, and are interrupted by some 50 introns. The exons coding for the amino- and carboxy-terminal ends of the triple-helical region also code for the telopeptides and part of the propeptides. The rapid accumulation of procollagen mRNA in embryonic connective tissue requires that the excision of the multiple noncoding regions in the primary transcript be both precise and efficient. Thus, we can now add a multistep assembly of coding regions in the nucleus to the multistep assembly of collagen chains (triple-helix formation, glycosylation, cleavage of propeptides, fibrillogenesis and cross-linking) in the cytoplasm and Golgi apparatus as a requirement for the production of collagen fibrils. One may wonder why the synthesis of this major component of the extracellular matrix has evolved into such an exquisitely complicated process.

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15. Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. *Nature* **283**, 220–224 (1980).
16. Rogers, J. & Wall, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1877–1879 (1980).
17. Konkel, D. A., Maizel, J. V. Jr & Leder, P. *Cell* **18**, 865–873 (1979).
18. Heilig, R., Perrin, F., Gannon, F., Mandel, J. L. & Chambon, P. *Cell* **20**, 625–637 (1980).
19. Cochet, M. *et al. Nature* **282**, 567–574 (1979).
20. Woo, S. L. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3688–3692 (1978).
21. Miller, H. I., Konkel, D. A. & Leder, P. *Nature* **275**, 772–774 (1978).
22. Sharp, P. A. *Cell* **23**, 643–646 (1981).
23. Gage, L. P. & Manning, R. F. *J. biol. Chem.* **255**, 9444–9450 (1980); Manning, R. F. & Gage, L. P. *J. biol. Chem.* **255**, 9451–9457 (1980).
24. Seidman, J. G., Leder, A., Nau, M., Norman, B. & Leder, P. *Science* **202**, 11–17 (1978).
25. Lehrach, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 5417–5421 (1978).
26. Dodgson, J. B., Strommer, J. & Engel, J. D. *Cell* **17**, 879–887 (1979).
27. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201–5205 (1980).
28. Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
29. Fietzek, P. P. & Rexrodt, F. W. *Eur. J. Biochem.* **59**, 113–118 (1975).
30. Highberger, J. H., Kang, A. H. & Gross, J. *Biochemistry* **10**, 610–616 (1971).
31. Fuller, F. & Boedtker, H. *Biochemistry* **20**, 996–1006 (1981).

## LETTERS TO NATURE

### The 26.13 MHz absorption line in the direction of Cassiopeia A

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It has recently been suggested<sup>1</sup> that the absorption line  $\nu = 26.13$  MHz that we had detected<sup>2</sup> in the direction of Cassiopeia A might be a recombination line due either to carbon (C631 $\alpha$ ) present in H I clouds or to heavier elements of hot gas. Observations reported here taken in February 1981 suggest that the low-frequency attenuation in the spectrum of Cas A is due to the presence of a cold cloud of ionized carbon.

Recombination lines can be defined by measuring adjacent lines, for example 630 $\alpha$  and 632 $\alpha$  in the present case. We carried out similar observations during the summer of 1978 although these were hampered by high interference. We have re-examined a series of the spectra near the line C630 $\alpha$  ( $\nu = 26.250$  MHz) and averaged the maximum number of recordings not deteriorated by interference (total time 3 h—see Fig. 1). The resulting spectrum contains an absorption line whose frequency ( $\nu = 26.254$  MHz) agrees well with the calculation for the C630 $\alpha$  at the local standard of rest and with allowances made

for the line-of-sight velocities in the Perseus arm. The intensity is close to that of the line  $\nu = 26.13$  MHz. Neither the H630 $\alpha$  nor the H631 $\alpha$  line are observed. In February 1981 we performed more observations. Figure 2 shows the spectrum near the frequency 25.04 MHz, showing the line C640 $\alpha$  in absorption.

Thus, it seems reasonable to assume that we have observed a recombination line of carbon. However, more measurements are planned during the summer of 1981 (the most favourable time for observing Cas A) to check this assumption.

The decametric band spectral measurements were also carried out in other galactic directions, over a wide range of frequencies including both the <sup>14</sup>N lines and Hn $\alpha$ , Hen $\alpha$  and Cn $\alpha$  recombination lines which were not detected. The upper estimate of their intensities was  $\Delta T_L/T_c < 3 \times 10^{-4}$  (ref. 3).

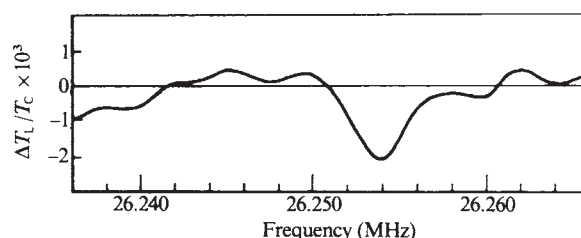


Fig. 1 The spectrum near the line C630 $\alpha$ . The frequency corrected for motion of the Earth. Frequency resolution, 4 kHz; storage time, 3 h.

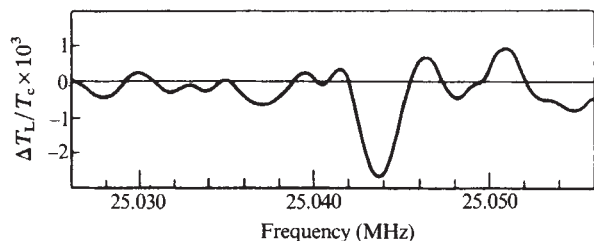


Fig. 2 The spectrum near the line C640α. The frequency corrected for motion of the Earth. Frequency resolution, 2 kHz; storage time, 10 h.

If the observed absorption line were really due to ionized carbon, it would yield unexpected results.

The frequency dependence of the carbon line intensity in the direction of Cas is interesting. Considering only frequencies  $\nu < 1,000$  MHz where the brightness temperature of Cas A is essentially higher than the electron temperature of the cloud, we have for the relative intensity of the recombination line<sup>4,5</sup>

$$\frac{\Delta T_L}{T_c} = -\tau_L^* \left[ 1 - \frac{kT_e}{h\nu} \left( 1 - \frac{B_n}{B_{n+1}} \right) \right] e^{-\tau_c} B_n$$

where  $\tau_L^*$  is the equilibrium optical depth at the recombination line;  $\tau_c$  the optical depth in the continuum; and  $B_n$  a factor accounting for the deviation of the  $n$ th level from the local thermodynamic equilibrium conditions.

Figure 3 illustrates the relative intensity of carbon recombination lines as a function of frequency for  $N_e = 0.1 \text{ cm}^{-3}$ ,  $T_e = 50 \text{ K}$  and measure of emission,  $ME = 0.07 \text{ pc cm}^{-6}$ . The values of  $\Delta B_n/B_n$  for  $n > 500$  are an extrapolation of earlier data<sup>5</sup>:  $\Delta B_n/B_n = 7 \times 10^{-6}$  at 25 MHz (solid line) and  $\Delta B_n/B_n = 2 \times 10^{-5}$  at 25 MHz (dashed line).

Figure 3 shows that the lines observable in the decametre waveband are absorption lines; at 26 MHz the results which fit our measurements ( $\Delta T_L/T_c \sim 2 \times 10^{-3}$ ) are similar to those reported by Blake *et al.* Near 40 MHz the lines are extremely weak. They can be observed as emission lines at frequencies  $\sim 150$  MHz, reaching quite high intensities (to  $10^{-2}$ ). This is also true for recombination lines of hydrogen with the same parameters as above. However, because the latter have never been detected either in the direction of Cas A (refs 3, 6, 7) or in

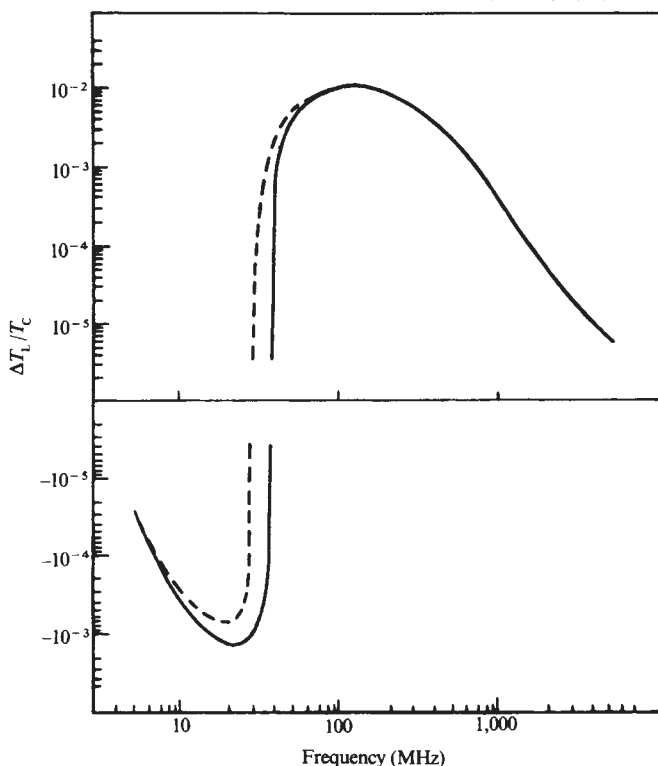


Fig. 3 The calculated relative intensities of carbon recombination lines versus frequency.

other directions, it suggests a low degree of ionization of the hydrogen clouds  $\zeta_H < 10^{-17} 1/c$ . The band of analysis used in the search for the H300α (ref. 6) and H352α lines (ref. 7) covered some carbon lines. The present calculations show the detectability of such lines for carbon clouds compared with the parameters given by Blake *et al.*<sup>1</sup>. In the spectrum of Shaver *et al.*<sup>7</sup>, several values have been excluded near the C352α line ( $\nu = 150.214 \text{ MHz}$ ), as allegedly deteriorated by interference. If the theoretical data given by Shaver<sup>4,5</sup> are correct, the carbon line should be observed in emission, with an intensity almost one order of magnitude higher than the detection threshold achieved. In this regard, observations in the frequency range 100–200 MHz would be of interest where emission recombination lines can be detected in cold clouds if they are sufficiently amplified by induced radiation.

Until recently, the decrease in the intensity of low-frequency radiation from Cas A was explained by the presence on the line of sight of a H II region, with the electron concentration  $N_e \sim 1 \text{ cm}^{-3}$ , temperature  $T_e \sim 5,000 \text{ K}$  and  $ME \sim 100 \text{ pc cm}^{-6}$  (refs 7, 8). However, almost the same amount of free-free absorption can be caused in this direction by a cloud of carbon with  $N_e = 0.1 \text{ cm}^{-3}$ ,  $T_e = 50 \text{ K}$  and  $ME = 0.07 \text{ pc cm}^{-6}$ . Hence, the low-frequency attenuation in the spectrum of Cas A might well be due to the presence of a cold cloud of ionized carbon.

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1. Blake, D. H., Crutcher, R. M. & Watson, W. D. *Nature* **287**, 707–708 (1980).
2. Konovalenko, A. A. & Sodin, L. G. *Nature* **283**, 360–361 (1980).
3. Konovalenko, A. A. & Sodin, L. G. *Soviet astr. J. Lett.* **5**, 663–664 (1979).
4. Shaver, P. A. *Pramana* **5**, 1–29 (1975).
5. Shaver, P. A. *Astr. Astrophys.* **49**, 1–16 (1976).
6. Casse, J. L. & Shaver, P. A. *Astr. Astrophys.* **61**, 805–808 (1977).
7. Shaver, P. A., Pedlar, A. & Davis, R. D. *Mon. Not. R. astr. Soc.* **177**, 45–50 (1976).
8. Braude, S. Ya. & Tchayevski, E. V. *Izv. Vuzov, Radiofiz.* **5**, 211–215 (1962).

## High-sensitivity detection of negative ions in the stratosphere

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Mass spectrometer composition measurements of stratospheric negative ions were first made by Arnold and Henschen<sup>1</sup> using a balloon-borne instrument. These initial measurements, which were taken at an altitude of 36.5 km, revealed that the major ion species are  $\text{NO}_3^-(\text{HNO}_3)_n$  and  $\text{HSO}_4^-(\text{H}_2\text{SO}_4)_n(\text{HNO}_3)_m$ . The suggestion that the unexpected  $\text{HSO}_4^-$  cores are formed from  $\text{NO}_3^-$  cores by a reaction involving stratospheric sulphuric acid vapour<sup>1</sup> was strongly supported by laboratory measurements of the relevant ion-molecule reactions by Viggiano and colleagues<sup>2</sup>. Meanwhile, major instrumental refinements in balloon-borne ion mass spectrometers, including increased mass range, sensitivity and mass resolution, have resulted in the detection of more massive  $\text{HSO}_4^-(\text{H}_2\text{SO}_4)_n$  ions and in the extension of the height range from a thin layer around 35 km to a region extending from 23 to 39 km (refs 3, 4). Here we report *in situ* negative ion composition measurements of even higher sensitivity which led to the detection of previously unobserved ion species.

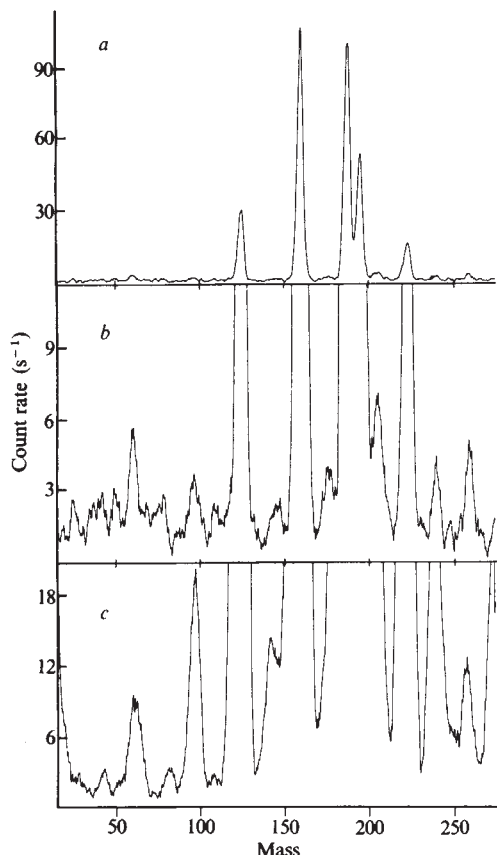
The instrument, which has been described in detail elsewhere<sup>3,5–7</sup>, consists of a cryogenically (liquid neon) pumped quadrupole mass filter with single ion detection by a channel electron multiplier operating in a pulse-saturated ion counting mode. The ions were drawn into the pumped chamber by applying 10 V to the front plate. The mass filter, which has a mass range of 0–276 AMU, can be operated in various modes differing in mass resolution and sensitivity. It also includes a so-called integral mode which allows the detection of ions having masses  $>276 \text{ AMU}$  (see ref. 3). The ion detection limit of the present instrument is about  $1 \text{ ion cm}^{-3}$ , which corresponds



to a fractional ion abundance of  $5 \times 10^{-4}$  at 34 km. This 20-fold improvement in sensitivity over previous instruments was obtained by a simplification of the ion optical system—an enhanced ion signal was obtained by removing ion lenses and focusing elements between the inlet orifice and the quadrupole. An additional increase in counting efficiency was achieved by placing a grid directly in front of and at equipotential with the detector channel multiplier.

The present measurements were taken on 26 September 1980 during a daytime flight over southwestern France. Figure 1 shows data obtained at the peak or 'float' altitude of 34 km at noon local time. The US Standard Atmosphere temperature for this location and time is 234 K (ref. 8). An additional measure of temperature was obtained by considering the proton hydrate positive ion spectrum taken during the same flight<sup>9</sup>. Taking the measured ratio of adjacent proton hydrates,  $H^+(H_2O)_3/H^+(H_2O)_4$ , a typical water mixing ratio of 4 p.p.m. and the laboratory thermodynamic data of Kebarle<sup>10</sup>, we calculate a temperature of 237 K at 34 km. As the US Standard Atmosphere temperature represents average stratospheric conditions and because our method for temperature determination has not been independently corroborated, we assume an average of 235 K with error bars of 5 K.

Two sample spectra are shown in Fig. 1. The spectrum of highest resolution or A-mode spectrum (Fig. 1a) and the expanded version (Fig. 1b) is the sum of 100 mass sweeps of 11 s each; the peak width at half height is  $\sim 4$  AMU. The spectrum shown in Fig. 1c is the sum of 50 sweeps with the mass spectrometer in the B-mode; in this mode a larger ion sensitivity



**Fig. 1** Sample negative ion spectra measured at 34 km. The total scan time was 1,100 s and the dark count rate was 1 c.p.s. *a* shows major ions with the mass spectrometer set at its highest resolution (A-mode); *b* is the same A-mode spectrum expanded to show minor peaks; *c* was taken with mass spectrometer set for lower resolution but higher sensitivity (B-mode). The large apparent peak at the lower end of the B-mode scale is due to the integral signal at zero mass of the quadrupole<sup>3</sup>. Counting statistics were improved by subjecting raw data, divided into 1,000 channels along the mass scale, to a running mean averaging where the 'window' was smaller than the peak width.

**Table 1** Mass numbers, ion identifications and fractional ion count rates of negative ions at 34 km

| Mass (AMU) | Ion                                                                                                                                                                        | Fractional ion count rate (%) |
|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| Group I    |                                                                                                                                                                            |                               |
| 125 ± 0.5  | NO <sub>3</sub> <sup>-</sup> ·HNO <sub>3</sub>                                                                                                                             | 1.8                           |
| 160 ± 0.5  | HSO <sub>4</sub> <sup>-</sup> ·HNO <sub>3</sub>                                                                                                                            | 6.5                           |
| 188 ± 0.5  | NO <sub>3</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub>                                                                                                              | 6.2                           |
| 195 ± 0.5  | HSO <sub>4</sub> <sup>-</sup> ·H <sub>2</sub> SO <sub>4</sub>                                                                                                              | 3.3                           |
| 223 ± 0.5  | HSO <sub>4</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub>                                                                                                             | 0.9                           |
| Group II   |                                                                                                                                                                            |                               |
| 61 ± 1     | NO <sub>3</sub> <sup>-</sup> , CO <sub>3</sub> <sup>-</sup>                                                                                                                | 0.1–0.4                       |
| 97 ± 1     | HSO <sub>4</sub> <sup>-</sup> , NO <sub>3</sub> <sup>-</sup> ·HCl, CO <sub>3</sub> <sup>-</sup> ·HCl                                                                       |                               |
| 174 ± 2    | NO <sub>3</sub> <sup>-</sup> ·HNO <sub>3</sub> ·HOCl, HSO <sub>4</sub> <sup>-</sup> ·HNO <sub>3</sub> ·H <sub>2</sub> O                                                    |                               |
| 206 ± 2    | NO <sub>3</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub> ·H <sub>2</sub> O                                                                                            |                               |
| 240 ± 2    | NO <sub>3</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub> ·HOCl, HSO <sub>4</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub> ·H <sub>2</sub> O                      |                               |
| 259 ± 2    | HSO <sub>4</sub> <sup>-</sup> ·H <sub>2</sub> SO <sub>4</sub> ·HNO <sub>3</sub>                                                                                            |                               |
| Group III  |                                                                                                                                                                            |                               |
| 26 ± 2     | CN <sup>-</sup>                                                                                                                                                            | 0.05–0.1                      |
| 43 ± 2     | CN <sup>-</sup> ·H <sub>2</sub> O                                                                                                                                          |                               |
| 80 ± 2     | NO <sub>3</sub> <sup>-</sup> ·H <sub>2</sub> O, CO <sub>3</sub> <sup>-</sup> ·H <sub>2</sub> O                                                                             |                               |
| 109 ± 2    | NO <sub>3</sub> <sup>-</sup> ·HNO <sub>2</sub>                                                                                                                             |                               |
| 133 ± 2    | HSO <sub>4</sub> <sup>-</sup> ·HCl                                                                                                                                         |                               |
| 142 ± 2    | NO <sub>3</sub> <sup>-</sup> ·HNO <sub>3</sub> ·H <sub>2</sub> O                                                                                                           |                               |
| 148 ± 2    | HSO <sub>4</sub> <sup>-</sup> ·HOCl                                                                                                                                        |                               |
| 211 ± 2    | HSO <sub>4</sub> <sup>-</sup> ·HNO <sub>3</sub> ·HOCl, HSO <sub>4</sub> <sup>-</sup> ·H <sub>2</sub> SO <sub>4</sub> ·H <sub>2</sub> O                                     |                               |
| 131 ± 2    | HSO <sub>4</sub> <sup>-</sup> ·H <sub>2</sub> SO <sub>4</sub> ·HCl                                                                                                         |                               |
| 248 ± 2    | HSO <sub>4</sub> <sup>-</sup> ·H <sub>2</sub> SO <sub>4</sub> ·HOCl                                                                                                        |                               |
| 273 ± 3    | HSO <sub>4</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub> ·HOCl,<br>HSO <sub>4</sub> <sup>-</sup> ·H <sub>2</sub> SO <sub>4</sub> ·HNO <sub>3</sub> ·H <sub>2</sub> O |                               |

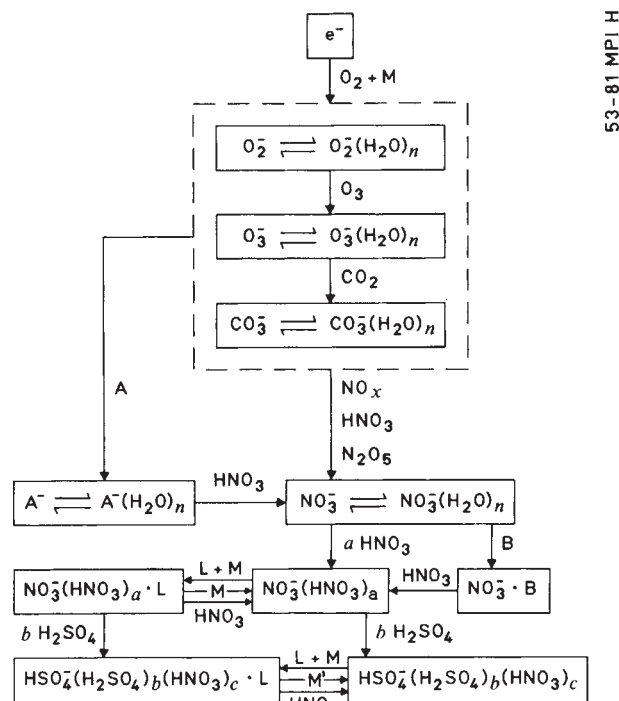
Ions are separated into groups according to peak count rates. Adjustment in the fractional ion count rate was made for sulphuric acid clusters which are out of mass range of the instrument (see ref. 3).

is obtained at the expense of mass resolution (the peak width is  $\sim 7$  AMU). The A-mode allows an unambiguous mass identification for the major peaks ( $\pm 0.5$  AMU). For the peaks near the detection limit the total signal is of the order of 100 ion counts per AMU; the dark current is about 20 counts per AMU interval (A-mode). Thus, poor counting statistics and associated poor peak shape result in a mass uncertainty of  $\pm 2$  AMU.

It can be seen that in the low mass region of Fig. 1 peak crowding and poor counting statistics hamper peak identification in the A-mode; in the B-mode, although the peak widths are larger, the minor peaks are more readily identified. In the high mass region, on the other hand, the A-mode spectrum is more informative. This is due to extended flanks of the dominant acid peaks in the B-mode spectrum which in many cases overlap and mask the much less intense minor peaks. Another feature evident in Fig. 1 is the change in abundance of some of the minor peaks between the A- and B-modes, notably the relative abundances of mass  $61 \pm 1$  to  $97 \pm 1$  AMU and mass  $240 \pm 2$  to  $259 \pm 2$  AMU. Although this difference may result partly from statistical fluctuations in the least sensitive A-mode, it could also be due to temporal and/or spatial variations in the ambient ion composition.

Table 1 is a compilation of observed mass peaks identified from spectra like those in Fig. 1 together with tentative ion assignments. Comparison of the integral mode and the resolving mode spectra reveals that most of the ambient ions have masses  $> 276$  AMU and thus could not be analysed. This result is consistent with other recent *in situ* observations<sup>3,4</sup>.

To aid discussion, the ions listed in Table 1 have been separated into three groups according to ion signal strength. Group I consists of ions with fractional ion count rates  $> 0.01$  and whose mass numbers can be identified to within  $\pm 0.5$  AMU. These ions belong to the families  $NO_3^-(HNO_3)_a$  and  $HSO_4^-(H_2SO_4)_b(HNO_3)_c$  and have all been observed previously with fractional ion count rates similar to those reported here<sup>1,3,4</sup>. The minor ions fall into two groups: those with fractional ion count rates between 0.001 and 0.004 are placed in group II and



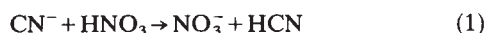
**Fig. 2** Stratospheric negative ion reaction scheme including proposed minor ion species.  $L$  refers to the ligands  $H_2O$ ,  $HCl$  and  $HOCl$ . Hydrate equilibria are established quickly due to the relatively high water concentrations.

those with count rates between 0.005 and 0.001 in group III. In general, the peaks in group III are the most poorly defined and contain the largest uncertainty; nevertheless, their count rates are significantly above the background signal. The relatively higher count rates and better peak shapes of group II ions allow a mass number determination of  $\pm 1$  AMU compared with  $\pm 2$  AMU for the group III ions. In some cases, such as with  $174 \pm 2$  AMU, the mass position cannot be accurately determined because of the presence of an adjacent major peak.

As with the major negative ions, the minor ions seem to contain  $NO_3^-$  and  $HSO_4^-$  cores. There are, however, at least two cases where this does not apply; the core ion for the masses 26 and 43 is tentatively identified as  $CN^-$ . Also, due to the limited accuracy in mass number identification, we cannot exclude a contribution from  $CO_3^-$  and  $CO_3^- \cdot H_2O$  to the peaks at  $61 \pm 1$  and  $79 \pm 2$  AMU. The other minor ions seem to contain ligand molecules attached to  $NO_3^-$  and  $HSO_4^-$  other than  $HNO_3$  and  $H_2SO_4$ . These ligands are tentatively identified as  $H_2O$ ,  $HCl$ ,  $HNO_2$  and  $HOCl$ . Although certainly not unique, these identifications seem reasonable; such Brønsted–Lowry acids are known to bond strongly to negative ions<sup>11</sup>.  $H_2O$ , the weakest of the acids, is also a likely ligand due to its large abundance. Arijis *et al.*<sup>4</sup> have also recently reported several minor ions containing  $H_2O$  ligands.

As a certain ambiguity exists in the assignment of cluster ions on the basis of mass alone, we have also used physicochemical arguments to obtain ion identifications. We expect water as a second or third ligand, for example, only if the precursor cluster ion has a relatively high abundance. This is due to the relatively weak  $H_2O$  ligand bonds and to the general decrease in free energy of bonding with increasing ligand number<sup>12</sup>. Thus, the ion at  $142 \pm 2$  AMU could well be  $NO_3^- \cdot HNO_3 \cdot H_2O$  or the ion at  $206 \pm 2$  AMU  $NO_3^-(HNO_3)_2 \cdot H_2O$ ; the precursor clusters in these cases,  $NO_3^- \cdot HNO_3$  and  $NO_3^-(HNO_3)_2$ , are major ions in the spectrum. On the other hand, the ion at 248 AMU is not given the structure  $HSO_4^- \cdot H_2SO_4 \cdot HCl \cdot H_2O$  because of the low abundance of the  $HSO_4^- \cdot H_2SO_4 \cdot HCl$  ion. The masses which cannot be decided on this basis are given in Table 1 with multiple cluster ion assignments.

The core ion  $CN^-$  should react with  $HNO_3$  via



as the electron affinity of  $CN$  ( $3.8 \text{ eV}$ )<sup>13</sup> is smaller than that of  $NO_3$  ( $4.0 \text{ eV}$ )<sup>14</sup> and the bond energy of  $HCN$  exceeds that of  $HNO_3$  (ref. 15). The absence of ions containing  $CN^-$  cores with  $HNO_3$  ligands also suggests that  $HNO_3$  reacts with the core rather than attaches to it.

Proposed reaction schemes for stratospheric negative ions include the core ion  $CO_3^-$ , but do not take  $CN^-$  reactions into account<sup>16</sup>. Due to the large electron affinity of  $CN$ , the  $CN^-$  core may be formed from  $O_2^-$ ,  $O_3^-$  or  $CO_3^-$  cores by reactions involving a  $CN$  compound, probably  $HCN$ . By assuming that  $CN^-$  cores are lost primarily by a reaction involving  $HNO_3$  and that their abundances reach a kinetic steady state, we estimate the  $HCN$  abundances as being of the same order as the  $HNO_3$  abundance. This qualitative result is consistent with recent IR absorption measurements of Coffey *et al.*<sup>17</sup>.

Ions containing the ligands  $H_2O$ ,  $HCl$ ,  $HNO_2$  and  $HOCl$  would be expected to be lost by  $HNO_3$  switching reactions because, for the cluster ion sizes considered, a ligand bonds more strongly the higher its gas phase acidity<sup>10</sup>. As the gas phase acidity of  $HNO_3$  exceeds those of  $H_2O$ ,  $HCl$ ,  $HNO_2$  and  $HOCl$  (refs 15, 18), nitric acid should exoergically displace these ligands. It is also conceivable that ions containing such weakly bound ligands as  $H_2O$  are lost primarily by thermal dissociation (see Fig. 1).

Fragmentation during ion sampling is known to be minimal for the strongly bound clusters with sulphuric and nitric acid ligands<sup>6,7</sup>; but an important question is whether the weakly bound  $H_2O$  ligands are affected by fragmentation. This question might be answered by considering the hydration equilibria of the observed ions. However, for a hydration equilibrium to obtain, as opposed to a dynamic steady state, the dominant loss process for a hydrate ion  $A^- \cdot H_2O$  must be thermal dissociation and not switching with an ambient species of higher gas phase acidity. Using the forward rate constants and association free energies for several negative ion hydrations given by Fehsenfeld and Ferguson<sup>19</sup>, we calculate thermal dissociation rates which are typically an order of magnitude faster than nitric acid switching reactions calculated with an upper limit gas collision rate constant. Thus, if the hydrate ions are not significantly fragmented during ion sampling, the measured fractional abundances for  $A^- \cdot H_2O$  and  $A^-$  ions should reflect ion hydration equilibria of the form

$$\frac{[A^- \cdot H_2O]}{[A^-][H_2O]} = k_{eq} = \exp(-\Delta G/RT)$$

From the measured fractional ion count rates and a typical 4 p.p.m.v. water mixing ratio<sup>20</sup>, we calculate the free energy differences,  $\Delta G$ , shown in Table 2. These values are compared with laboratory  $\Delta G$ s for the hydrations of  $CN^-$  and  $NO_3^-$ ; in the absence of data for the hydration of  $NO_3^- \cdot HNO_3$  and  $NO_3^-(HNO_3)_2$ , we use available data for the hydration of  $NO_3^- \cdot H_2O$  and  $NO_3^-(H_2O)_2$ . In view of the uncertainty in atmospheric parameters such as temperature and water concentration, the good agreement shown in Table 2 is fortuitous and no rigorous conclusions can be drawn from it; nevertheless, we feel that it provides further support for our ion

**Table 2** Free energies of hydration derived from observed fractional ion count rates and water number density of  $8 \times 10^{11} \text{ cm}^{-3}$

| $A^-$                   | Association order | $-\Delta G$ (kcal mol <sup>-1</sup> ) |            |
|-------------------------|-------------------|---------------------------------------|------------|
|                         |                   | Derived from data                     | Literature |
| $CN^-$                  | 1                 | $8.3 \pm 1$                           | 9.1*       |
| $NO_3^-$                | 1                 | $8.0 \pm 1$                           | 7.9*, 8.7† |
| $NO_3^- \cdot HNO_3$    | 2                 | $6.7 \pm 1$                           | 7.3‡       |
| $NO_3^-(HNO_3)_2$       | 3                 | $6.7 \pm 1$                           | 6.0‡       |
| $HSO_4^- \cdot H_2SO_4$ | 2                 | $6.4 \pm 1$                           | —          |

\* Ref. 12.

† Ref. 28.

‡  $\Delta G$  for the addition of  $H_2O$  to  $NO_3^-(H_2O)_n$  of the same association order.



identifications as well as for the contention that the hydrates are not severely fragmented during ion sampling.

Ions containing the acid ligands HCl, HNO<sub>2</sub> and HOCl may be formed either by three-body association or by switching of the corresponding ligand molecules. If association occurred at a gas collision rate and if HNO<sub>3</sub> switching was indeed the major sink for these ions, a steady-state treatment would yield an estimate for the abundances of HCl and HOCl of 0.1 to 1 times that of the HNO<sub>3</sub> concentration and for HNO<sub>2</sub> as roughly equal to HNO<sub>3</sub>.

Although stratospheric IR absorption measurements show the HCl abundance to be comparable with that of HNO<sub>3</sub> at 34 km (ref. 21), HOCl has not been observed. Calculations based on laboratory rate data of HOCl reactions and photolysis predict HOCl abundances which may be as large as that of HNO<sub>3</sub> (refs 22–24 and P. Crutzen, personal communication). We feel, therefore, that the identification of HCl and HOCl ligands attached to stratospheric negative ions is at least plausible.

Nitrous acid is another trace gas which has not been observed in the stratosphere. An important source of this species is the reaction  $\text{NO} + \text{OH} + \text{M} \rightarrow \text{HNO}_2 + \text{M}$  and the main loss path is photolysis<sup>25</sup>. Photolysis of HNO<sub>2</sub>, however, is much more efficient than that of HNO<sub>3</sub> so that one would not expect the abundance of HNO<sub>2</sub> to be as high as is apparent from the present measurements (P. Crutzen, personal communication). Clearly, further atmospheric as well as model investigations of this species are needed.

Finally, we note that HNO<sub>3</sub> concentrations may be derived from the present measurements by a method which relies on the existence of a kinetic steady state with respect to the nitric and sulphuric acid cluster ions taken as a whole. The common precursor of these clusters is the NO<sub>3</sub><sup>-</sup> hydrate family—seen here for the first time—and the common sink is ion-ion recombination. The steady-state continuity equation is simply solved for the HNO<sub>3</sub> concentration; it is

$$[\text{NO}_3^-(\text{H}_2\text{O})_n]k[\text{HNO}_3] = [\text{NO}_3^-(\text{HNO}_3)_a + \text{HSO}_4^-(\text{H}_2\text{SO}_4)_b(\text{HNO}_3)_c]\alpha n_+$$

where  $k$  represents the rate constants for HNO<sub>3</sub> association and switching with NO<sub>3</sub><sup>-</sup>(H<sub>2</sub>O)<sub>n</sub>,  $\alpha$  is the ion-ion recombination coefficient, and  $n_+$  is the total positive ion concentration. Taking  $k = 10^{-9} \text{ cm}^3 \text{ s}^{-1}$  (Fehsenfeld *et al.*<sup>14</sup> report a lower limit of  $5 \times 10^{-10} \text{ cm}^3 \text{ s}^{-1}$ ),  $\alpha = 10^{-7} \text{ cm}^3 \text{ s}^{-1}$  (refs 6, 7) and  $n_+ = 2,000 \text{ cm}^{-3}$ , we obtain a HNO<sub>3</sub> concentration of  $7 \times 10^7 \text{ cm}^{-3}$ . If, alternatively, the HNO<sub>3</sub> concentration is derived from the abundance ratio of NO<sub>3</sub><sup>-</sup>(HNO<sub>3</sub>)<sub>2</sub> and NO<sub>3</sub><sup>-</sup>·HNO<sub>3</sub> (equilibrium method, see ref. 26), one obtains values which depend strongly on the set of thermodynamic data used. The data of Davidson *et al.*<sup>27</sup> ( $\Delta H = -18.3 \text{ kcal mol}^{-1}$  and  $\Delta S = -22.1$  entropy unit) give  $7 \times 10^7 \text{ cm}^{-3}$ , the data of Lee *et al.*<sup>27</sup> ( $\Delta H = -17.7 \text{ kcal mol}^{-1}$ ,  $\Delta S$  not measured, we calculate  $-20.1$  e.u. from  $\Delta G$  values of Davidson *et al.*) give  $9.6 \times 10^7 \text{ cm}^{-3}$ , and those of Wlodek *et al.*<sup>29</sup> ( $\Delta H = -16.3 \text{ kcal mol}^{-1}$ ,  $\Delta S = -23.1$  e.u.) give  $9 \times 10^9 \text{ cm}^{-3}$ . Comparison of the above inferred HNO<sub>3</sub> concentrations reveals agreement between the steady-state and equilibrium methods if the thermodynamic data of Davidson *et al.* are used. These values are also in reasonable agreement with recent model calculations of P. Crutzen (personal communication) which give an HNO<sub>3</sub> concentration of  $7 \times 10^8 \text{ cm}^{-3}$  at 34 km. A word of caution is in order, however, concerning the degree of agreement in the above derived HNO<sub>3</sub> concentrations. Considering the uncertainties in input parameters such as temperature and kinetic and thermodynamic data, this close agreement is certainly fortuitous; it does, however, provide grounds for optimism about the use of ion measurements to deduce stratospheric nitric acid concentrations. Laboratory kinetic and thermodynamic studies of the relevant ions as well as improved *in situ* measurements are needed to improve our understanding of minor negative ions. Planned atmospheric measurements are aimed at an extended height range and an unambiguous mass identification of the minor ions.

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1. Arnold, F. & Henschen, G. *Nature* **257**, 521–522 (1978).
2. Viggiano, A. A., Perry, R. A., Albritton, D. L., Ferguson, E. E. & Fehsenfeld, F. C. *J. geophys. Res.* **85**, 4551–4555 (1980).
3. Viggiano, A. A. & Arnold, F. *Geophys. Res. Lett.* (in the press); *Planet Space Sci.* (in the press).
4. Arijis, E., Nevejans, D., Frederick, P. & Ingels, J. *Geophys. Res. Lett.* (in the press).
5. Arnold, F., Böhringer, H. & Henschen, G. *J. geophys. Lett.* **5**, 653–656 (1978).
6. Arnold, F., Henschen, G. & Ferguson, E. E. *Planet. Space Sci.* **29**, 185–193 (1981).
7. Arnold, F., Fabian, R., Ferguson, E. E. & Joos, W. *Planet. Space Sci.* **29**, 195–203 (1981).
8. *U.S. Standard Atmosphere Suppl.* (U.S. Government Printing Office, Washington DC, 1966).
9. Henschen, G. & Arnold, F. *Nature* **291**, 211–213 (1981).
10. Kebarle, P. A. *Rev. phys. Chem.* **28**, 445–476 (1977).
11. Yamdagni, R. & Kebarle, P. *J. Am. chem. Soc.* **93**, 7139–7143 (1971).
12. Payzant, J. D., Yamdagni, R. & Kebarle, P. *Can. J. Chem.* **49**, 3308–3314 (1971).
13. Janousek, B. K. & Brauman, J. I. in *Gas Phase Ion Chemistry* Vol. 2 (ed. Bowers, M. T.) Ch. 10 (Academic, New York, 1979).
14. Fehsenfeld, F. C., Howard, C. J. & Schmeltekopf, A. L. *J. chem. Phys.* **63**, 2835–2841 (1975).
15. Stull, D. R. & Prophet, H. (eds) *JANAF Thermodynamical Tables 37* (National Standards Reference Data Series, U.S. National Bureau of Standards, 1971).
16. Ferguson, E. E., Fehsenfeld, F. C. & Albritton, D. L. in *Gas Phase Ion Chemistry* Vol. 1 (ed. Bowers, M. T.) Ch. 2 (Academic, New York, 1979).
17. Coffey, M. T., Mankin, W. G. & Cicerone, R. J. *Science* (submitted).
18. Rosenstock, H. M., Draxl, K., Steiner, B. W. & Herron, J. T., *J. phys. chem. Ref. Data* **6** (1977).
19. Fehsenfeld, F. C. & Ferguson, E. E. *J. chem. Phys.* **61**, 3181–31393 (1974).
20. Elsaesser, H. W., Harries, J. E., Kley, D. & Penndorf, R. *Planet. Space Sci.* **28**, 827–835 (1980).
21. Ackerman, M. J. *atmos. terr. Phys.* **41**, 723–733 (1979).
22. Prasad, S. S., Jaffe, R. L., Whitten, R. C. & Turco, R. P. *Planet. Space Sci.* **26**, 1017–1026 (1976).
23. Glasgow, L. C., Jesson, J. P., Filkin, D. L. & Miller, C. *Planet. Space Sci.* **27**, 1047–1054 (1979).
24. Molina, M. J., Ishiwata, T. & Molina, L. T. *J. phys. Chem.* **84**, 821–826 (1980).
25. Platt, U., Perner, D., Harris, G. W., Winer, A. M. & Pitts, J. N. *Jr Nature* **285**, 312–314 (1980).
26. Arnold, F., Fabian, R., Henschen, G. & Joos, W. *Planet. Space Sci.* **28**, 681–685 (1980).
27. Davidson, J. A., Fehsenfeld, F. C. & Howard, C. J. *Int. J. mass Spectrom. Ion Phys.* **9**, 17–29 (1977).
28. Lee, N., Keesee, R. G. & Castleman, A. W. Jr *J. chem. Phys.* **72**, 1089–1094 (1980).
29. Wlodek, S., Luczynski, Z. & Wincel, H. *Int. J. mass Spectrom. Ion Phys.* **35**, 39–46 (1980).

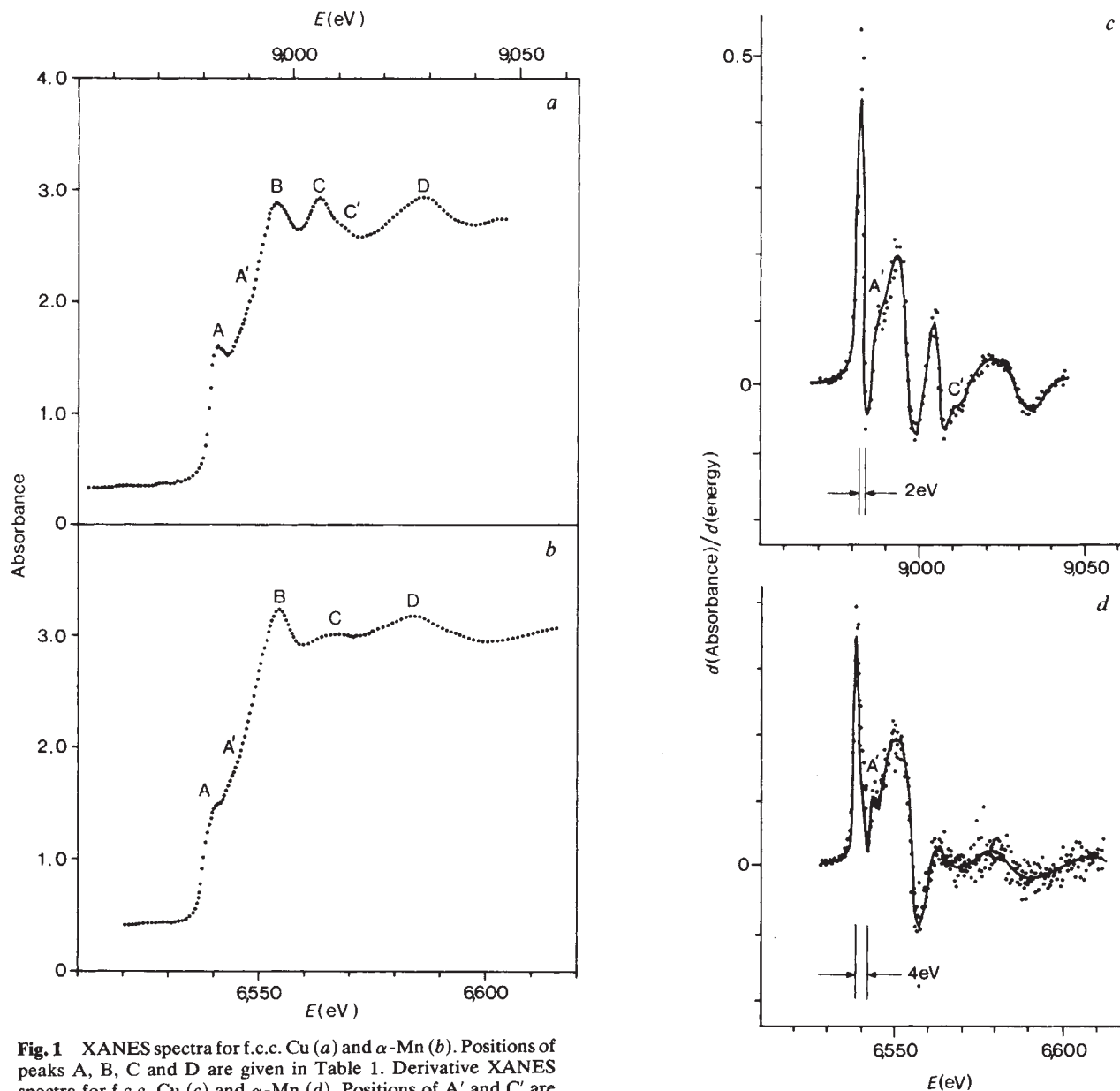
## Near-edge X-ray absorption spectra for metallic Cu and Mn

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The measurement of X-ray absorption fine structure of metals—both in the extended region (EXAFS) as well as in the near edge region (XANES)—has been widely discussed (see refs 1–6 for Cu and refs 7–9 for Mn). The recent availability of intense X-ray fluxes from storage rings has usually been exploited for EXAFS leaving the XANES often with poorer resolution than earlier work performed on conventional sources (for example, compare the near edge structure for copper in ref. 1 with refs 3 or 6). In addition, whilst the theory and analysis of EXAFS is relatively well-established<sup>2,10</sup>, a theory for the strong scattering regime near to the absorption edge has only recently been developed<sup>11</sup>. We report here the first high resolution XANES spectra for Cu and Mn which were performed at the SRS storage ring at Daresbury. Although both metals have close-packed structures consisting of atoms of similar size their local atomic structure is different in detail. Significant differences are found in their respective XANES reflecting the sensitivity of this region of the X-ray absorption fine structure to the local atomic structure. Spectra for the two metals have been analysed using the new multiple scattering formalism. This is a real space calculation and unlike a conventional band structure approach it does not require structural periodicity but works from the local arrangement of atoms.

K-edge absorption spectra for f.c.c. Cu (8.98 keV) and  $\alpha$ -Mn (6.54 keV) are presented in Fig. 1a, b with the respective



**Fig. 1** XANES spectra for f.c.c. Cu (a) and  $\alpha$ -Mn (b). Positions of peaks A, B, C and D are given in Table 1. Derivative XANES spectra for f.c.c. Cu (c) and  $\alpha$ -Mn (d). Positions of A' and C' are given in Table 1. A cubic spline is included to guide the eye.

derivative spectra in Fig. 1c, d. The raw unsmoothed data are presented demonstrating the excellent signal-to-noise ratio obtained. Measurements were made at room temperature in transmission using 10- $\mu$ m metallic foils supplied by Goodfellow Metals. The Cu foil was 99.99% pure and the Mn foil 98.7% pure (encapsulated in polyethylene). A Si 220 channel cut crystal diffracting vertically was used to monochromatize the white beam. Argon-filled ion chambers were used for detection in the conventional arrangement<sup>12</sup>. The 1-mm high entrance slits at 16 m from the source subtended an angle  $\Delta\theta$  of 13 arc s. This is two or three times larger than the angle subtended by the source or the width of the crystal rocking curve at these wavelengths and therefore defines the instrumental resolution. For the Si 220 crystal this is given by  $5.7 \times 10^{-5} \tan \theta \Delta\theta$  which for the Cu and Mn edges equals 1.5 and 0.8 eV respectively. ( $\theta$  is the Bragg angle given by  $\lambda = 3.84 \sin \theta$  Å). The 2-eV wide peak on the leading edge of the Cu spectrum (Fig. 1a) demonstrates the resolution achieved. The instrumental resolution was chosen to match the inherent spectral broadening. This derives from the finite lifetimes of the photoemitted electron and the core hole. Of these the core hole lifetime makes the principal contribution when the energy of the photoelectron is low. For Mn and Cu the inverse hole lifetime broadening is between 1 and 2 eV.

The spectra displayed in Fig. 1 were obtained during the commissioning period of the SRS when the machine was running with 10 mA of electron beam at an energy of 1.5 GeV. The comparatively low energy meant harmonic spectral contamination was minimized. However, coupled with the low beam current and the narrow entrance slits ( $1 \times 10$  mm), this reduced the photon flux at the sample<sup>13</sup> to  $\sim 5 \times 10^7$  photons  $s^{-1}$ . Data collection times resulted in typically  $10^8$  photons per point giving an integrated photon limited signal-to-noise ratio of  $10^4$ . This value which compares well with the point-to-point noise recorded in the featureless parts of the spectra and indicates that there was no significant contribution from the detection system or from the residual scattered radiation in the vicinity of the ion chambers. For the SRS running at 2 GeV and 200 mA an improvement of at least an order of magnitude in signal-to-noise ratio is anticipated.

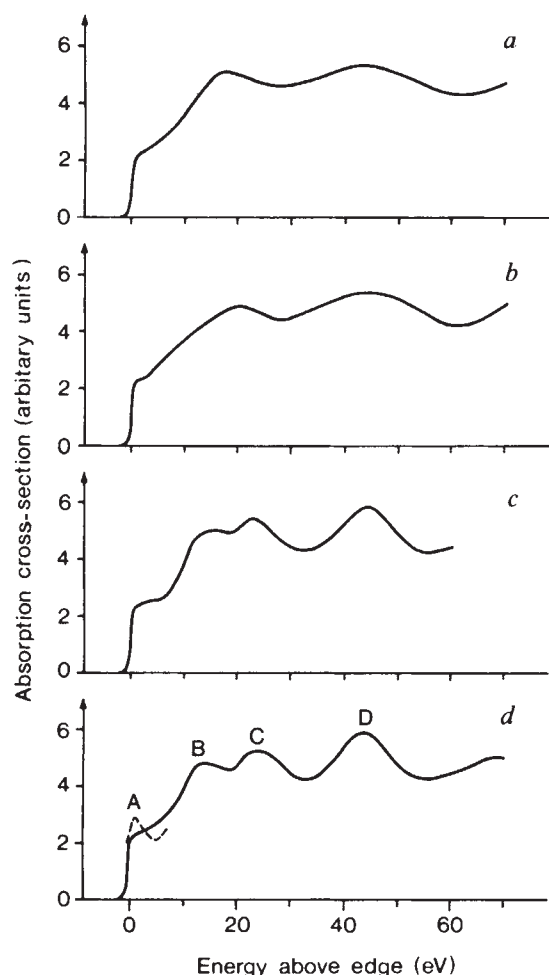
There are four strong peaks in the first 60 eV of fine structure for f.c.c. Cu. These are marked A, B, C and D in Fig. 1a, b. There is also some finer structure evident in the derivative spectrum (for example, A' and C' in Fig. 1c, d). The relative peak positions are listed in Table 1. Similar features also occur in the  $\alpha$ -Mn spectrum but the amplitudes are weaker on the whole (Fig. 1a, b) and the precise positions (Table 1) are different. This becomes clear by comparing the derivative spectra (Fig. 1c, d) where the similarity between f.c.c. Cu and  $\alpha$ -Mn only holds over the first



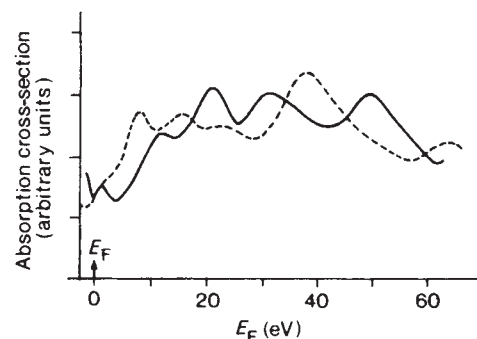
20 eV. The atomic volumes of copper and manganese are 11.7 and 12.1 Å<sup>3</sup> respectively and both have close-packed structures<sup>14</sup>. The differences in XANES almost certainly stem from the differences in local structure as other authors have concluded<sup>15</sup>. We will return to this point later.

Basically most fine structure in X-ray absorption spectra results from the scattering of the photo-emitted electron between the absorbing atom and those atoms surrounded it. The EXAFS at high energies ( $\geq 50$  eV above the absorption edge) is well described by single scattering theory<sup>10</sup>. In the XANES region close to the edge multiple scattering of the photoelectron plays an increasingly important part. As a result XANES in principle is sensitive to higher order atomic correlations<sup>11</sup> in addition to the pairwise correlations obtainable from EXAFS (or from X-ray or neutron diffraction, all of which are weak scattering probes). This means the fine structure is not simply interpretable as a combination of Fourier components—one for each shell of atoms<sup>9</sup>. Recently, Durham *et al.*<sup>11</sup> extended the single scattering treatment of EXAFS<sup>4</sup> to incorporate multiple scattering. In this approach the local density of states (the local electronic band structure<sup>16,17</sup>) is built up by considering explicitly the relevant electron scattering paths within a finite portion of the system. Moreover in our theory the X-ray absorption cross-section is calculated in a real space representation, thereby removing the requirement, in band structure calculations, for relatively simple periodic structures.

Multiple scattering theory for XANES has been used successfully to predict the *K*-edge structure of f.c.c. Ag



**Fig. 2** Multiple scattering calculations for different cluster sizes of f.c.c. Cu; a, 1 shell; b, 2 shells; c, 3 shells; d, 4 shells. These consist of 12 atoms at 2.55 Å (1st shell), 6 atoms at 3.61 Å (2nd shell), 24 atoms at 4.42 Å (3rd shell) and 12 atoms at 5.09 Å (4th shell). The broken line in curve d represents the results of removing the broadening altogether in the vicinity of the edge.



**Fig. 3** Multiple scattering calculations for Mn in hypothetical b.c.c. (solid curve) and f.c.c. (dashed curve) structures, each with the same atomic volume as  $\alpha$ -Mn. Four shells of neighbour atoms are included in both calculations, and the Fermi level,  $E_F$ , is an estimate consistent with band structure calculations<sup>20</sup>.

(25.5 keV)<sup>11</sup>. The near edge structure of f.c.c. Cu shown in Fig. 1 is qualitatively similar to, but rather more pronounced than, that of Ag, and accordingly provides a more decisive test of the theory. Figure 2 shows a sequence of calculations for f.c.c. clusters starting with one shell and running up to four shells. Clearly the final calculation for a 55-atom cluster gives quite good agreement with experiment (Fig. 1). The four main peaks A, B, C and D are all predicted and their positions are accurate practically to within the experimental spectral resolution of  $\pm 1.5$  eV (Table 1). The relative amplitudes of the peaks as well as their individual asymmetries are also reproduced. Compared with experiment, however, the amplitude of the theoretical fine structure oscillations is approximately double what it should be. Whilst the calculated spectra have been broadened by 2.7 eV to match the inverse hole lifetime, no allowance has been made for the energy dependent inverse electron lifetimes, shake-up/shake-off effects or thermal disorder—all of which will decrease the amplitude of the fine structure oscillations. Our results are in good agreement with the APW band structure calculations of Wakoh and Kubo<sup>15</sup> (see Table 1). The rather similar *K*-edge spectrum of Ni has been investigated by Szmulowicz and Pease<sup>18</sup> using an APW calculation) who also discuss the origin of the main low-energy structures.

The XANES calculations for different sizes of cluster shown in Fig. 2 emphasize the sensitivity of this region to many shells of atoms. For instance, the four peaks of f.c.c. Cu are only reproduced when at least the third shell of 24 atoms at 4.42 Å is included. EXAFS is also sensitive to these outer shells, but this region of the absorption fine structure is dominated by the scattering effects of the first shell of 12 atoms at 2.55 Å (ref. 19).

The allotropes of Mn have extremely distorted structures of which  $\alpha$ -Mn is the usual form<sup>14</sup>. The overall packing density in  $\alpha$ -Mn is close to that in the b.c.c. structure but the local structure

**Table 1** Peaks measured in the near edge structure of f.c.c. Cu and  $\alpha$ -Mn\* and the calculated positions for f.c.c. Cu using multiple scattering theory† with the APW band structure values‡

|                       | A (eV) | A' (eV) | B (eV) | C (eV) | C' (eV) | D (eV) |
|-----------------------|--------|---------|--------|--------|---------|--------|
| f.c.c. Cu             |        |         |        |        |         |        |
| Experiment            | 2.(4)  | 5.(1)   | 14.(0) | 24.(8) | 28.(3)  | 45.(6) |
| Theory (present work) | 1.(5)  |         | 14.(6) | 24.(4) |         | 44.(2) |
| Theory (ref. 15)      | 2.(8)  |         | 14.(3) | 23.(2) |         |        |
| $\alpha$ -Mn          |        |         |        |        |         |        |
| Experiment            | 3.(6)  | 5.(7)   | 17.(0) | 28.(2) |         | 45.(8) |

All peak positions are taken relative to the first maxima in the derivative spectra. The literature values for the first maxima for Cu and Mn are 8,980.3 and 6,538.0 eV respectively<sup>21</sup>.

\* From Fig. 1a, b.

† From ref. 11 and Fig. 2.

‡ From Fig. 1 of ref. 15.

shows few similarities. In particular there are four distinct first coordination shells containing between 12 and 16 atoms. The calculated XANES for Mn in hypothetical b.c.c. and f.c.c. structures with the same atomic volume as  $\alpha$ -Mn is shown in Fig. 3. The obvious differences between the two calculations demonstrates their sensitivity to local structure. On the other hand the f.c.c. Mn calculation shows clear similarities with the f.c.c. Cu calculations, but contains slightly more structure. In the same way the b.c.c. Mn calculation is qualitatively similar to the calculations of Wakoh and Kubo<sup>15</sup> on b.c.c. V and Fe. Comparison with experiment in Fig. 1 shows, however, that neither the b.c.c. nor f.c.c. Mn calculations agree well with the spectrum of  $\alpha$ -Mn. Although there are experimental similarities

between  $\alpha$ -Mn and Cu XANES, the amplitude of the oscillations are smaller for  $\alpha$ -Mn; for instance, peak A in the Cu spectrum becomes a shoulder for Mn. Evidently the quasi-disordered nature of the local structure in  $\alpha$ -Mn tends to wash out the sharp features of a more symmetrical structure. Preliminary multiple scattering calculations for the first shell of  $\alpha$ -Mn display a significant variation in the fine structure for different inequivalent sites, the amplitude of which diminishes when an average is taken. More detailed calculations will be published later.

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1. Beeman, W. W. & Friedman, H. *Phys. Rev.* **56**, 392–405 (1939).
2. Stern, E. A. *Phys. Rev.* **B10**, 3027–3037 (1974).
3. Martens, G., Rabe, P., Schwenter, N. & Werner, A. *Phys. Rev.* **B17**, 1481–1488 (1978).
4. Salem, S. I. & Hall, V. L. *J. Phys. F: Metal Phys.* **10**, 1627–1629 (1980).
5. Vlaic, G., Bart, J. C. J. & Cavigiolo, W. *Chem. Phys. Lett.* **76**, 453–459 (1980).
6. Eisenberger, P. & Lengler, B. *Phys. Rev. B* **22**, 3551–3562 (1980).
7. Hanson, H. & Beeman, W. W. *Phys. Rev.* **76**, 118–121 (1949).
8. Vaingankar, A. S., Khasbaddar, B. V. & Patil, R. N. *J. Phys. F: Metal Phys.* **9**, 2301–2305 (1979).
9. Bianconi, A. in *EXAFS for Inorganic Systems* (eds Garner, D. & Hasnain, S. S.) 13–22 (Daresbury Research Report SCI/R17/EXP, 1981).
10. Lee, P. A. & Pendry, J. B. *Phys. Rev.* **B11**, 2795–2811 (1975).
11. Durham, P. J., Pendry, J. B. & Hodges, C. H. *Solid State Commun.* **38**, 159–162 (1981).
12. Brown, G. S. & Doniach, S. in *Synchrotron Radiation Research* (eds Winick, H. & Doniach, S.) 353–385 (Plenum, New York, 1980).
13. Poole, J. H. *Daresbury Laboratory Technical Memorandum*, DL/SRF/TM 4 (1976).
14. Wyckoff, R. N. G. *Crystal Structures* (Wiley, New York, 1964).
15. Wakoh, S. and Kubo, Y. *Jap. J. appl. Phys.* **17**, (S17-2), 193–196 (1978).
16. Kostroun, V. O., Fairchild, W., Kukkonen, C. A. & Wilkins, J. W. *Phys. Rev.* **B13**, 3268–3271 (1978).
17. Muller, J. E., Jepsen, O., Andersen, O. K. & Wilkins, J. W. *Phys. Rev. Lett.* **40**, 720–722 (1978).
18. Szmulowicz, F. & Pease, D. M. *Phys. Rev.* **B17**, 3341–3355 (1978).
19. Gurman, S. J. & Pendry, J. B. *Solid St. Commun.* **20**, 287–290 (1976).
20. Moruzzi, V. L., Janak, J. F. & Williams, A. R. in *Calculated Electronic Properties of Metals* (Pergamon, New York, 1978).
21. Bearden, J. A. & Burr, A. F. *Rev. mod. Phys.* **39**, 125–142 (1967).

## Solid-state reduction of iron in olivine—planetary and meteoritic evolution

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Iron-nickel metallic particles have been reported in meteorites<sup>1</sup> and lunar<sup>2–5</sup> and terrestrial<sup>6,7</sup> rocks. The origin of these metallic particles is not unique as they may be formed by (1) condensation from a primordial solar nebula<sup>8</sup>; (2) crystallization from a melt; and (3) subsolidus reduction reactions under low oxygen or sulphur fugacity. We report here an electron microscopy study of the solid-state microstructural development in olivine single crystals ( $\text{Fo}_{92}$ ) in which half of the iron has been reduced to the metallic state by a gas–solid interaction in the temperature range 950–1,500 °C. The reaction,  $\text{Fo}_{92} \rightarrow \text{Fo}_{96} + \text{metallic Fe}(\text{Ni in solid solution}) + \text{pyroxene}$ , begins with a homogeneous transformation involving fine-scale metallic precipitates resembling Guinier–Preston zones<sup>9</sup>. The microstructure develops by the growth of the first-formed precipitates during an Ostwald ripening process<sup>9</sup> in which the precipitates located in the dislocation sub-boundaries develop in preference to precipitates in the subgrains. On the other hand, pyroxene is first observed to nucleate heterogeneously at pre-existing dislocations and its coarsening rate is more than an order-of-magnitude faster than that of the metallic phase. Besides the textural similarity of the observed microstructures with that reported for some of the lunar materials<sup>2</sup>, these results have important implications for the physical models of accretion of terrestrial planets, planetesimals and meteorites<sup>10</sup>, especially with respect to the distribution of siderophile elements. The rate of reaction observed here places constraints on models for the formation of the Earth's core by segregation of a metallic phase with or without reduction.

Single crystals of San Carlos olivine,  $\text{Fo}_{92}$ , were heated under controlled oxygen fugacity,  $f_{\text{O}_2}$ , to temperatures in the range 950–1,500 °C and the conductivity of the samples measured<sup>11</sup>. These results indicated that the time-dependent variation in electrical conductivity was associated with a change in the

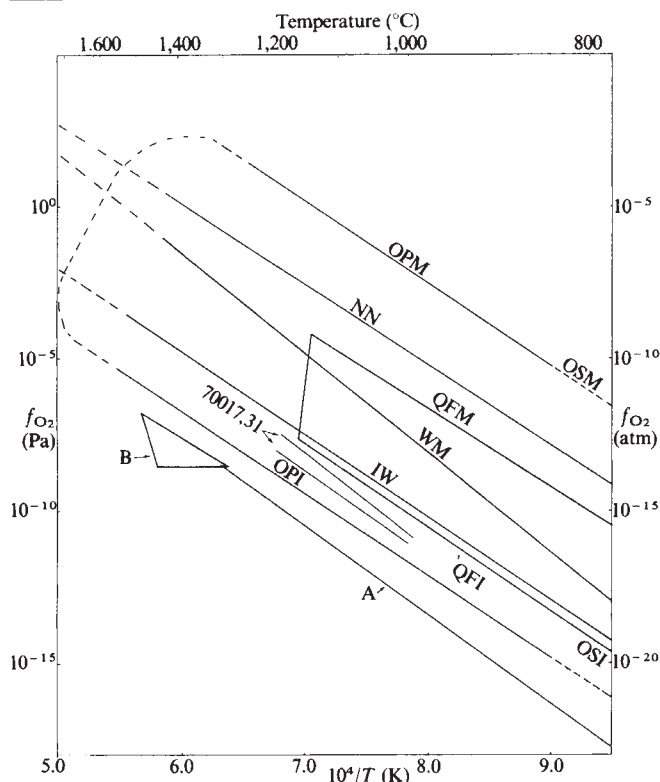
oxidation state of iron. One sample (B) that had experienced reducing conditions during conductivity measurements was selected for electron microscopy studies. In addition, sample A was heated in the experimental assembly without conductivity measurements to study the early stages of the reduction reaction—it spent 1 h at 1,400 °C, 3 h at 1,300 °C and 1 h at 1,200 °C in  $\text{CO}/\text{CO}_2$  mixtures defined by line A in Fig. 1. Sample B was at 1,500 °C for 1.5 h, 1,400 °C for 2 h and 1,300 °C for 21 h in  $\text{H}_2/\text{CO}_2$  mixtures defined by region B (Fig. 1). The conductivity of sample B decreased by 50% in the first 2 h but remained unaltered during the next 23 h.

Assuming that the conductivity decrease is due to Fe loss from the olivine<sup>11</sup>, then the reaction of olivine with the imposed  $f_{\text{O}_2}$  is completed within the first 2 h at these temperatures. Consequently, sample A should represent the earliest stages in the nucleation and growth of the metallic phase produced by the reduction reaction, while sample B would indicate the microstructural development in the coarsening process. To determine the growth rate parameters, all kinetic data were standardized to 1,300 °C. After the reaction, the composition of the matrix in sample B was  $\text{Fo}_{96}$ , while its initial nickel content of 0.33 wt% was reduced to a level that was undetectable. Because of the homogeneous distribution of the fine-scaled precipitates in sample A, it was not possible to determine the matrix composition.

The microstructure of sample A was variable. The first indications of the reduction process were the matrix strain-field contrast effects<sup>12</sup> shown in Fig. 2a. These effects were homogeneously distributed in this region of the sample and not markedly affected by the pre-existing dislocations. However, in other areas, the dislocations had precipitates attached to them (Fig. 2b). Using the energy dispersive system attached to the JEOL 200 C microscope, the precipitate was analysed as a pyroxene. Unfortunately no extra diffraction information was found in the fine beam (0.05  $\mu\text{m}$ ) microdiffraction pattern. Sample B contained 0.2–1.5  $\mu\text{m}$  blebs of iron–nickel (2 wt% Ni) distributed predominantly in dislocation sub-boundaries. These precipitates (M), kamacite, (identified by selected area electron diffraction), were located in the edge-dislocation sub-boundaries composed of  $b=[100]$  and  $b=[001]$  dislocations but were absent from cross-arrays of screw dislocations (Fig. 3a). Scanning electron microscopy indicated that the coarsening process for the metallic phase occurred throughout the bulk of the sample (Fig. 3b) and high-voltage microscopy (0.9 MeV) confirmed the distribution of the phase in thick sections (4–

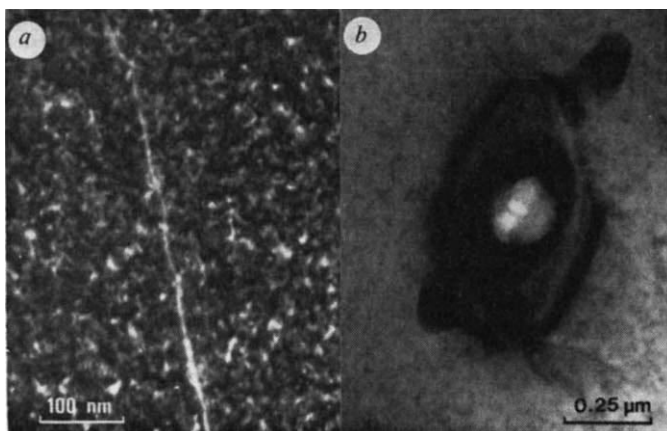
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**Fig. 1** Temperature versus  $f_{O_2}$  stability of olivine (after ref. 37) with 10% fayalite and pure fayalite, respectively compared with various oxygen buffers and lunar samples. OPM (olivine, pyroxene, magnetite) and OSM (olivine, silica, magnetite) delineate the oxidation of olivine with 10% fayalite. Reduction of olivine of that composition is indicated by OPI (olivine, pyroxene, iron) and OSI (olivine, silica, iron). For fayalite, the oxidation boundary is indicated by QFM (quartz, fayalite, magnetite) and QFI (quartz, fayalite, iron) (see ref. 34 for detailed discussion of these stability fields). The buffers NN (nickel–nickel oxide), WM (wüstite–magnetite), and IW (iron–wüstite) are shown for comparison. Also shown is the intrinsic  $f_{O_2}$  determined for lunar basalt<sup>35</sup> and the experimental paths A and B discussed in the text.

5  $\mu\text{m}$ ). However, the pyroxene that formed during the reduction reaction was more heterogeneously distributed than in sample A, and, when observed, had grown to precipitates larger than 5  $\mu\text{m}$ . It had a high density of planar faults lying in the (100) planes that produced streaks in the diffraction pattern; there were also clinoenstatite lamellae up to 0.1  $\mu\text{m}$  wide in the orthopyroxene<sup>13</sup>. Although the starting material had a dis-



**Fig. 2** *a*, A dark-field image of the fine-scale precipitates of Fe–Ni in the olivine matrix—the dislocation has not acted as a preferred nucleation site: sample A. *b*, A pyroxene precipitate located at a dislocation loop: sample A.

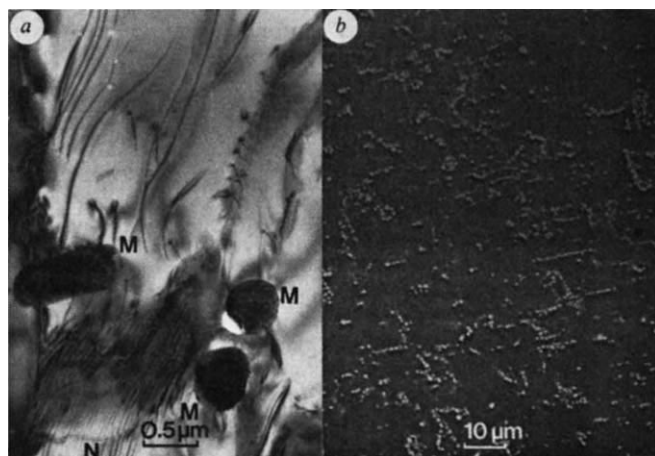
location density of  $4(\pm 2) \times 10^5 \text{ cm}^{-2}$  with widely spaced (100) and (001) dislocation sub-boundaries ranging from 50 to 100  $\mu\text{m}$ , sample B had an average subgrain size of 2.5  $\mu\text{m}$  and a free dislocation density of  $6(\pm 3) \times 10^8 \text{ cm}^{-2}$ .

Figure 4 illustrates what is postulated to be the coarsening process for the metallic phase. The precipitate ( $M_1$ ) within the subgrain is dissolving while the precipitate ( $M_2$ ) at the sub-boundary coarsens. The dissolution reaction at  $M_1$  involves predominantly lattice diffusion with some minor pipe diffusion along the attached dislocation while the growth process of  $M_2$  occurs by pipe diffusion down the dislocation in the sub-boundary. This is the most likely mechanism for the growth of the few larger iron–nickel particles observed in the SEM (Fig. 3*b*).

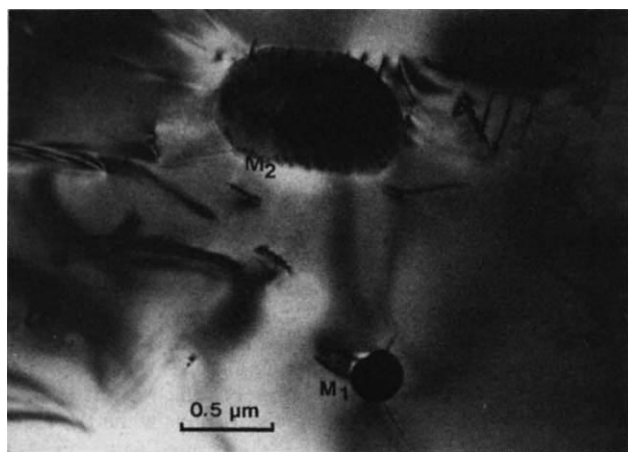
It is apparent that the nucleation and growth processes of the metallic and silicate phases are different. The earliest evidence of the pyroxene is at pre-existing dislocations while the matrix still shows the strain-contrast effects, assumed to be the iron–nickel pre-precipitates, similar to Guinier–Preston (GP) zones<sup>9</sup>. However, incoherency at the interphase boundaries soon develops<sup>14,15</sup> producing both interfacial and lattice dislocations.

The growth rate of the pyroxene is difficult to estimate but the coarsening rate of the metallic phase can be calculated from the conductivity measurements on sample B, using a diffusion-controlled model for precipitate growth<sup>16</sup> and Fe diffusivity in olivine<sup>17</sup>. The effects of pressure are important for reactions that may occur in the mantle. Pressure influences the diffusivity of Fe through the  $PV$  term in the general diffusion equation<sup>18</sup>, where  $V$  is the activation volume for the rate-controlling step. One experimentally-determined value of  $V$  in olivine, based on the dislocation climb-recovery rate, is 11  $\text{cm}^3$  (ref. 19). This dislocation climb-related process is probably controlled by oxygen diffusivity. In another study of the rheological behaviour of olivine in the upper mantle, a value of 7  $\text{cm}^3$  was used, assuming that both power-law creep and diffusional flow were controlled by oxygen ion diffusivity<sup>18</sup>. There are no reported values of  $V$  for Fe diffusion in olivine, so a range of values—0, 1, 7 and 11  $\text{cm}^3$ —has been used to calculate precipitate sizes. Pressure and temperature influence the stability of olivine. To depths of 400 km (150 kbar), olivine is stable but with increasing depth, it transforms according to the following<sup>20</sup>: at 400 km olivine transforms to the  $\beta$ -polymorph; in the range 400–650 km the  $\beta$ -phase will transform to the spinel phase,  $\gamma$ ; at  $\sim 650$  km the spinel most likely transforms, by a disproportionation reaction, into the perovskite and rocksalt structures which seem to persist deep into the lower mantle.

The temperature of the olivine to  $\beta$ -phase transformation is estimated to be 1,650  $^\circ\text{C}$ , based on the mantle temperature distribution<sup>10</sup>. Taking these  $P, T$  conditions to be the optimum



**Fig. 3** Iron–nickel precipitates (M) in sample B. *a*, The precipitates are located in the edge dislocation sub-boundaries but they are absent from the cross-array of screw dislocations at N. *b*, SEM showing the clustering of the metallic precipitate as well as the crystallographic alignment: sample B.



**Fig. 4** The smaller metallic precipitate at  $M_1$  lies within the subgrain while the larger precipitate,  $M_2$ , is attached to the (100) edge dislocation sub-boundary—according to the postulated coarsening mechanism,  $M_1$  is dissolving while  $M_2$  is growing: sample B.

for the growth of the iron (nickel) precipitates in olivine the precipitate sizes expected after  $10^9$  yr coarsening are 20, 15, 2 and 1 mm using the values of  $V$  given above. These calculations, based on our experimental results, use the Gibbs–Thompson equation<sup>14,16</sup> assuming: (1) the precipitate/matrix interfacial free-energy and, (2) the equilibrium solubility of Fe in the matrix (for large precipitates), are constant over the  $P$ – $T$  ranges used. Although there are no data for the Fe diffusivity in the  $\beta$ ,  $\gamma$ , perovskite and rocksalt structures, it is unlikely that the coarsening rates will differ by more than one or two orders of magnitude from the values calculated according to the olivine data, provided appropriate  $P$ ,  $V$  and  $T$  values are used in the diffusion equation. For example, based on the olivine data, in  $10^9$  yr, at 2,000 km (corresponding to 2,500 °C and 100 GPa–1 Mbar), the precipitate size would be about 5 mm for a  $V$  of  $1 \text{ cm}^3$ . One other variable is the  $f_{\text{O}_2}$ . Intrinsic  $f_{\text{O}_2}$  measurements<sup>21</sup> on spinels from peridotites indicate a wide range from just above the iron–wustite buffer to the OPI buffer (Fig. 1).  $f_{\text{O}_2}$  will certainly influence the diffusion coefficient but as with Ni diffusion in NiO (ref. 22) the effects may be small for very low  $f_{\text{O}_2}$ .

Although solid-state diffusion may not lead to any large particles of iron–nickel, it has recently been proposed that the incorporation of Fe (and Ni) in refractory metal particles in the Allende meteorite occurs as a postnebular condensation–reduction reaction<sup>23</sup>. In addition, the condensates from a nebular gas accrete to form planetesimals and ultimately the planets. Unless the accreting body is completely molten, it will be necessary to transfer iron (and nickel) from a solid silicate phase into the metallic phase which may be molten (see below); in addition, other light elements including sulphur, oxygen, silicon and hydrogen may diffuse into the metallic phase. The mechanism of formation (nucleation) and coalescence (coarsening) of the metal will be critical steps in the segregation/sinking stage of the metallic phase into the formation of the core, regardless of whether the overall accretional processes are described as homogeneous<sup>10</sup> or heterogeneous<sup>24,25</sup>.

For a homogeneous transformation involving GP-like zone formation, the diffusivity pathways are exceedingly small, possibly no greater than 200 Å between zones. However, if the zones are now liquid instead of solid, the coarsening process would not involve the slow solid-state, Ostwald ripening process but a modified version of hydrodynamic dispersion in which the mass transfer occurs by molten iron convecting along subgrain and grain boundary channels. This mass transfer process has effective diffusivities several orders of magnitude greater than normal diffusivities<sup>26</sup>. In addition our observation shows that in the very early stages of the formation of metallic particles, nickel is preferentially removed from the silicate phase. To avoid the

excessive depletion of siderophile elements implied by such a process, advocates of the homogeneous accretional theory propose the sinking of large metallic ‘drops’ that originate close to the surface of the Earth<sup>27</sup> or at 1,500–2,000 km depths<sup>10</sup>. The former model implies that olivine would be the stable phase while in the later proposal the high-pressure polymorphs would be more appropriate. However, at such high pressures the ferrous ion in the silicate phase is expected to disproportionate into the ferric state plus metallic iron<sup>28</sup>. In any case, whether the formation of a metallic phase is by a reduction reaction or disproportionation, a coarsening reaction is implied and our observations for the solid state and those relating to the molten state<sup>29</sup>, indicate that large-sized precipitates would not form. A further complication arises because there is no agreement as to the relative abundances of siderophile elements (contrast refs 10 and 30), leaving the metal–silicate partitioning a highly controversial issue in models of Earth and Moon formation.

The boundary conditions imposed by the coarsening rates of solid and/or liquid precipitates in a solid silicate phase indicate that the evolution of the mantle and core involves a multi-stage processing of primitive material. Relicts of these primitive microstructures would be rare<sup>31</sup> because of subsequent modifications occurring during phase transformations<sup>32</sup> and mantle convection<sup>33</sup>. Although the solid-state growth rates reported here are insignificant for the large scale phenomena needed for core formation, they are certainly adequate to account for the micrometre size metallic phases and exsolution lamellae reported in lunar rocks<sup>2,34,35</sup> and meteorites<sup>36</sup>.

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1. Wasson, J. T. *Meteorites* (Springer, Berlin, 1974).
2. Haggerty, S. E. *Proc. 8th Lunar Sci. Conf.* 1809–1829 (1977).
3. Misra, K. M., Walker, B. M. & Taylor, L. A. *Proc. 7th Lunar Sci. Conf.* 2251–2266 (1976).
4. Brett, R. *Geochim. cosmochim. Acta* **40**, 997–1004 (1976).
5. Taylor, S. R. *Lunar Science: a Post-Apollo View* (Pergamon, New York, 1975).
6. Dick, H. J. B. *Earth planet. Sci. Lett.* **24**, 291–298 (1974).
7. Bassett, W. A., Bird, J. M., Weathers, M. S. & Kohlstedt, D. L. *Phys. Earth planet. Inter.* **23**, 255–261 (1980).
8. Grossman, L. *Geochim. cosmochim. Acta* **36**, 597–619 (1972).
9. Nicholson, R. B. & Davies, G. J. in *Structural Characteristics of Materials* (ed. Finiston, H. M.) 199–289 (Elsevier, Amsterdam, 1971).
10. Ringwood, A. E. *The Origin of the Earth and Moon* (Springer, Heidelberg, 1979).
11. Duba, A. & Nicholls, I. A. *Earth planet. Sci. Lett.* **18**, 59–64 (1973).
12. Lorimer, G. W. in *Electron Microscopy and Microanalysis of Crystalline Materials* (ed. Belk, J. A.) 29–55 (Applied Science, London, 1979).
13. Boland, J. N. *Contr. Miner. Petrol.* **47**, 215–222 (1974).
14. Champness, P. E. & Lorimer, G. W. in *Electron Microscopy in Mineralogy* (ed. Wenk, H. R.) 174–204 (Springer, Berlin, 1976).
15. Boland, J. N. in *Electron Microscopy*, Vol. 1 (eds Brederoo, P. & Boom, G.) 444–451 (7th European Congress E. M. Foundation, Leiden, 1980).
16. Martin, J. W. & Doherty, R. D. *Stability of Microstructure in Metallic Systems*, 155–244 (Cambridge University Press, London, 1976).
17. Buening, D. K. & Buseck, P. R. *J. geophys. Res.* **78**, 6852–6862 (1973).
18. Ashby, M. F. & Verral, R. A. *Phil. Trans. R. Soc. A* **288**, 59–93 (1978).
19. Kohlstedt, D. L., Nichols, H. P. K. & Hornack, P. J. *geophys. Res.* **85**, 3122–3130 (1980).
20. Liu, L.-G. in *The Earth: Its Origin, Structure and Evolution*, 177–202 (ed. McElhinny, M. W.) (Academic, New York, 1979).
21. Arculus, R. J. & Delano, J. W. *Geochim. cosmochim. Acta* **45**, 899–913 (1981).
22. Atkinson, A. & Taylor, R. I. *Phil. Mag.* **A39**, 581–595 (1979).
23. Blander, M., Fuchs, L. H., Horowitz, C. & Land, R. *Geochim. cosmochim. Acta* **44**, 217–223 (1980).
24. Ruff, L. & Anderson, D. L. *Phys. Earth planet. Int.* **21**, 181–201 (1980).
25. Smith, J. V. *Miner. Mag.* **43**, 1–81 (1979).
26. Domenico, P. A. A. *Rev. Earth planet. Sci.* **5**, 287–317 (1977).
27. Elasser, W. M. in *Earth Science and Meteorites* (eds Geiss, J. & Goldberg, E.) 1–30 (North Holland, Amsterdam, 1963).
28. Mao, H. K. & Bell, P. M. *Yb Carnegie Instn Wash.* **74**, 555–559 (1975).
29. Hofman, A. W. & Magaritz, M. *J. geophys. Res.* **82**, 5432–5440 (1977).
30. Wolf, R. & Anders, E. *Geochim. cosmochim. Acta* **44**, 2111–2124 (1980).
31. Bird, J. M. & Bassett, W. A. *J. geophys. Res.* **85**, 5461–5470 (1980).
32. Boland, J. N. *Terra Cognita*, Spring 1981, 84 (Abstract K1).
33. Anderson, D. L. *J. geophys. Res.* (in the press).
34. El Goresy, A. E., Taylor, L. A. & Ramdohr, P. *Proc. 3rd Lunar Sci. Conf.* 333–349 (1972).
35. McCallister, R. H. & Taylor, L. A. *Earth planet. Sci. Lett.* **17**, 357–364 (1973).
36. Grossman, L. A. *Rev. Earth planet. Sci.* **8**, 559–608 (1980).
37. Nitsan, U. *J. geophys. Res.* **79**, 706–711 (1974).



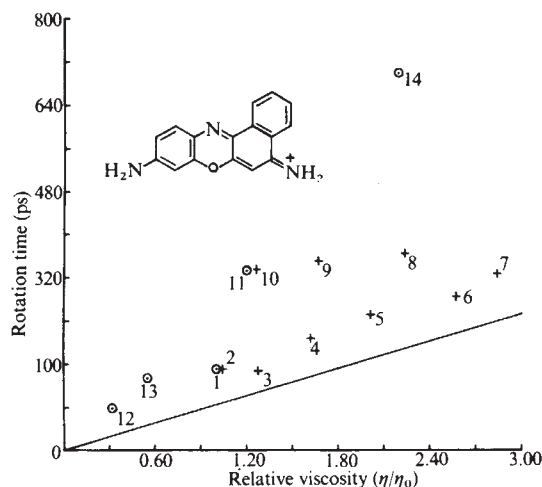
# Structural features in ethanol–water mixtures revealed by picosecond fluorescence anisotropy

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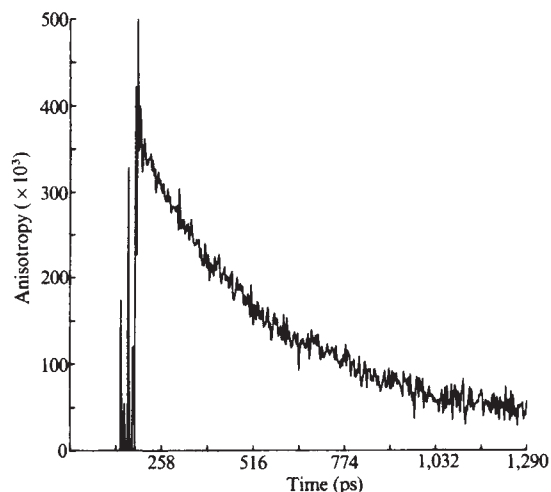
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When an ensemble of molecules is excited with polarized light an anisotropic orientational distribution with respect to the transition dipole moment is produced. This anisotropy can decay in time due to the rotational motion of the molecules and consequently leads to depolarization of the fluorescence<sup>1–6</sup>. The rate of this rotational motion has been successfully predicted from hydrodynamic theory. How much the rotational relaxation depends on molecular geometry and how much on specific solvent–solute interactions has been studied by picosecond spectroscopy<sup>1–6</sup> and other techniques<sup>7–9</sup>. In all cases so far reported, the rotational behaviour seems to be accounted for by the Debye–Stokes–Einstein (DSE) equation  $\tau_R = f/kT$ . This relates the rotational relaxation time  $\tau_R$  (inversely related to the rotational diffusion coefficient) to the frictional coefficient,  $f$ , which is proportional to the product of the shear viscosity, the molecular volume and a constant dependent on the ‘stick’ or ‘slip’ boundary conditions<sup>3,10,11</sup>. We report here, however, that large deviations from DSE behaviour have been observed in the rotational diffusion of the dye cresyl violet in ethanol–water mixtures. Different rotational relaxation times are observed in solutions of the same viscosity but differing composition. This behaviour can be rationalized using previously proposed models for water–ethanol mixtures.

Most information previously obtained from fluorescence depolarization data has been concerned with solvent–solute interactions rather than with the solvent itself<sup>5,6</sup>. Figure 1 shows a plot of the rotational relaxation time of the dye cresyl violet (3,7-diamino-1,2-benzophenoxazine chloride) against relative solvent viscosity ( $\eta/\eta_0$ ) for ethanol–water mixtures<sup>12</sup> (points 1–11 in order of increasing ethanol content) and for acetone, methanol and propan-1-ol (points 12–14 respectively). Also shown is the line calculated from DSE theory for cresyl violet at the ‘stick’ boundary condition. The molecular shape and volume were calculated from van der Waals’ radii. The composition ions of the solutions are shown in the legend to



**Fig. 1** Rotational relaxation time of cresyl violet (in ps) plotted against relative viscosity ( $\eta/\eta_0$ ) for water–ethanol mixtures (points 1–11) with ethanol mole fractions of 0.0, 0.002, 0.024, 0.051, 0.079, 0.132, 0.207, 0.470, 0.706, 0.950 and 1.0. Acetone, methanol and propan-1-ol are points 12–14 respectively. The solid line has the slope calculated for DSE behaviour of cresyl violet at the ‘stick’ boundary condition.



**Fig. 2** The decay of the fluorescence anisotropy  $r(t)$  for cresyl violet at 770 nm in the 0.470 mole fraction ethanol solution, calculated from the fluorescence intensity parallel ( $I_{\parallel}$ ) and perpendicular ( $I_{\perp}$ ) to the excitation polarization using the formula  $r(t) = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$ . The anisotropy is related to the rotational relaxation time  $\tau_R$  for a sphere by  $r(t) = r_0 \exp(-t/\tau_R)$ . For molecular shapes approximated to ellipsoids the formulae for  $r(t)$  given in refs 3 and 4 were used.

Fig. 1; all the experiments were carried out at 294K. The fluorescence of cresyl violet was measured with picosecond time resolution using a frequency up-conversion spectrometer<sup>6</sup>. The fluorescence anisotropy was calculated from the normalized fluorescence decays measured with polarization parallel and perpendicular to that of the exciting light. An example of the fluorescence anisotropy decay is shown in Fig. 2. The dye concentration was  $10^{-4}$  M in all the solutions and is low enough to avoid modification of the bulk properties of the solution.

The most noticeable feature of the rotational relaxation data are the dual values at identical solvent viscosities. We can identify two regions with which to explain the observations. First, in the high water region (points 1–7) DSE behaviour is observed and the gradient of the line of measured  $\tau_R$  against relative viscosity is the same as that predicted from the molecular volume. Second, after the maximum ethanol–water solution viscosity is reached (points 8–11) the rotational relaxation time decreases only slightly until the value for pure ethanol is reached, even though the relative viscosity changes by a factor of greater than 2. Ethanol–water mixtures show large positive deviations from Raoult's law and the viscosity is a maximum at a mole fraction of 0.25 ethanol. We have observed similar behaviour in other alcohols which will be reported elsewhere.

The difference in rotation time of cresyl violet between pure water and ethanol has previously been explained by the specific interaction of ethanol with the amino groups on cresyl violet<sup>6</sup>. This increases the effective volume of the molecule thus making molecular rotation slower. This argument, when extended to the other alcohols is the reason for their rotation times being well above the calculated ‘stick’ line, the solid line in Fig. 1. In water it seems that there is little interaction with the cresyl violet, presumably due to strong water–water interactions<sup>6</sup>. In the related dye oxazine-1 (3,7-bis(*N,N*-diethylamino) phenoxazine perchlorate) the amino groups are ethylated, hence no interaction can occur at this site with the alcohols; consequently, the rotation times in alcohols and alcohol–water mixtures fall just below the line calculated for ‘stick’ limit behaviour<sup>13</sup>.

Although the DSE theory was originally developed for large bodies moving in a continuum it seems, nevertheless, to be applicable to the description of small molecules moving in slightly smaller discrete solvents<sup>6,10</sup>. One reason for this would seem to be that the motion of the solvent is considerably faster than that of the dye molecules. For instance, a correlation time of 2.5 ps for the protons in water and 8 ps for the methyl protons in pure *t*-butanol has been reported<sup>14</sup>. The dye molecule, on the

other hand, rotates in  $\sim 150$  ps in pure water and 330 ps in pure ethanol. As the local environment around the molecule is changed many times during its rotational decay time the solvent effectively presents a continuum in which the solute moves. However, in the case of alcohol-water mixtures it seems that some structural features are maintained for long enough to affect the rotation of the solute.

Our results support models of 'hydrophobic hydration' deduced from thermodynamic and spectroscopic data<sup>15-17</sup>. It is suggested that in the region of increasing viscosity, up to  $\sim 0.2$  mole fraction ethanol, the ethanol may be considered to lie in interstices in a three-dimensional water 'structure'. The hydrophobic part of the ethanol is surrounded by 'cages' of water although it does not interact significantly with these surrounding molecules<sup>14</sup>; the hydroxyl group, however, replaces one of the framework water molecules. The altered reorientational behaviour of the water molecules on adding ethanol is due to the enhancement of water-water interactions between these cages. The water-ethanol interactions play a smaller part<sup>18</sup>. At higher alcohol concentrations, above  $\sim 0.2$  mole fraction ethanol the ethanol can no longer be contained in the basic water structure and this disintegrates; breaking of the water-water hydrogen bonds leads to a decrease in viscosity. A more detailed and comprehensive discussion of the properties of water-alcohol mixtures is given elsewhere<sup>16</sup>.

At low mole fraction of ethanol our results suggest (points 2-7, Fig. 1.) that the ethanol is surrounded by water as no specific interaction of the ethanol with the cresyl violet is observed; that is the variation of the rotational relaxation time with relative viscosity is that predicted for cresyl violet alone. Above the maximum viscosity when the ethanol is no longer contained in the water cages it is available for solvation of the cresyl violet. This is shown in the data by an increase in the measured rotational relaxation time when the solvent viscosity is decreasing (points 7-9). If the rotational diffusion rate at high ethanol concentrations were due to cresyl violet-ethanol interactions alone then  $\tau_R$  would be constant at 330 ps over the viscosity range.

The small variation in the rotation time over the viscosity range 2.8-1.2 indicates that in this composition range the cresyl violet is probing small pools of ethanol contained in the water. The slightly larger rotation times above 0.3 mole fraction (points 8 and 9) could be due to the small pool size at this composition as they are surrounded by water. This effect is analogous to that occurring for small pool sizes in reversed micelles where an increased viscosity is observed<sup>19</sup>. As the pools become larger the effect of the surrounding water is reduced and the rotation time tends towards that in pure ethanol. The possibility that the anisotropy decay probes free ethanol as well as water-ethanol cages was also considered. This would require the anisotropy to be a combination of two rotational decay times but analysis of the data in this manner will not support this supposition indicating that only a single species is present. Thus, due to its ability to interact preferentially with one component of the mixture cresyl violet can probe some of the structural features of alcohol-water mixtures that manifest themselves over a relatively long time, that is a few hundreds of picoseconds.

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1. Eistenthal, K. & Drexhage, K. *J. chem. Phys.* **51**, 5720-5721 (1969).
2. Chuang, T. & Eistenthal, K. *J. chem. Phys.* **57**, 5094-5097 (1972).
3. Tao, T. *Biopolymers* **8**, 609-632 (1969).
4. Fleming, G., Morris, J. & Robinson, G. *Chem. Phys.* **17**, 91-99 (1976).
5. von Jena, A. & Lessing, H. *Chem. Phys. Lett.* **78**, 187-193 (1981).
6. Beddard, G., Doust, T. & Porter, G. *Chem. Phys.* (in the press).
7. Bauer, D., Brauman, J. & Pecora, R. *J. Am. chem. Soc.* **96**, 6840-6843 (1974).
8. Pinnow, D., Candau, S. & Litowitz, T. *J. chem. Phys.* **49**, 347-362 (1968).
9. Hoel, D. & Kivelson, D. *J. chem. Phys.* **62**, 1323-1326 (1975).
10. Masters, A. & Madden, P. *J. chem. Phys.* **74**, 2450-2459 (1981); **74**, 2460-2465 (1981).
11. Hu, C. & Zwanzig, R. *J. chem. Phys.* **60**, 4354-4357 (1974).
12. *Handbook of Chemistry and Physics* 60th edn (CRC Press, 1980).
13. Fleming, G., Waldeck, D. & Beddard, G. *Il Nuovo Cimento* **63B**, 151-172 (1981).

14. Zeidler, M. in *Water—a Comprehensive Treatise* (ed. Franks, F.) Vol. 2, Ch. 10 (Plenum, New York, 1973).
15. Geiger, A., Rahman, A. & Stillinger, F. *J. chem. Phys.* **70**, 263-276 (1979).
16. Pratt, L. & Chandler, D. *J. Solut. Chem.* **9**, 1-17 (1980); *J. chem. Phys.* **67**, 3683-3688 (1977).
17. Franks, F. (ed.) *Water—a Comprehensive Treatise* Vol. 2, Chs 1, 5, 7, 9, 10 (1973); Vol. 4, Ch. 1 (1975) (Plenum, New York).
18. Franks, F. in *Water—a Comprehensive Treatise* Vol. 2, Ch. 1 (Plenum, New York, 1973).
19. Calvo-Perez, V., Beddard, G. & Fendler, J. *J. phys. Chem.* **85**, 2316-2319 (1981).

## Subduction of the Cocos plate in the Mid America Trench

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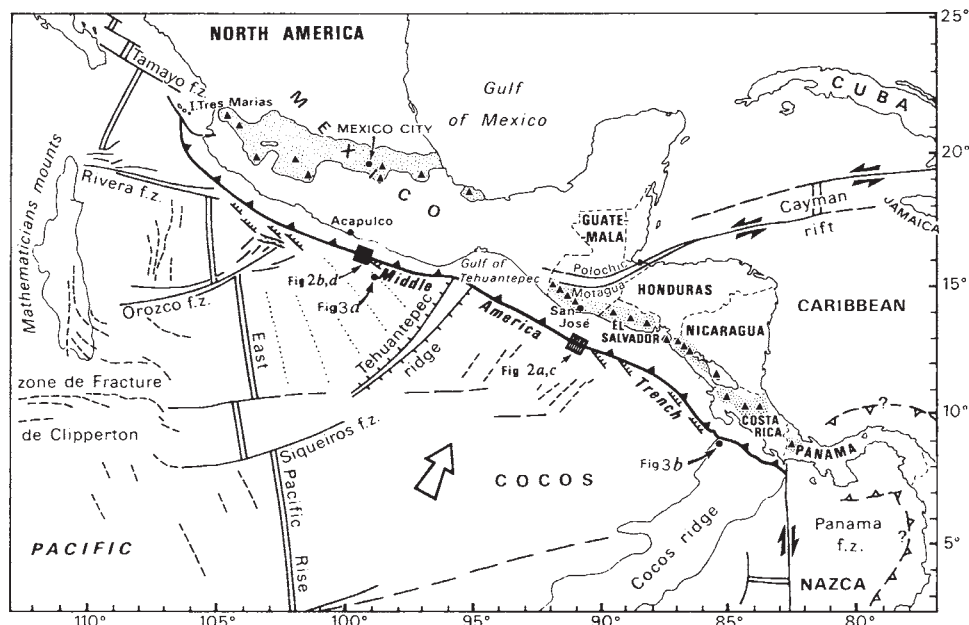
The Mid America Trench, off the Pacific coast of southern Mexico and central America, is the site of northeastward subduction of the Cocos oceanic plate under the North America and Caribbean plates<sup>1</sup> (Fig. 1). In Guatemala, the North America and Caribbean plates are separated by the Polochic-Motagua left-lateral strike-slip faults (Fig. 1). The trench itself appears to be divided into two distinct sections at its intersection with the Tehuantepec Ridge. To the south the margin of the trench is wide and consists of a well developed shelf basin<sup>2</sup> bounded on the east by a huge volcanic cordillera parallel with the trench axis at a distance of  $\sim 200$  km. In contrast, to the north, the margin is narrow and devoid of shelf basin; landwards, the trans-Mexican volcanic belt trends oblique to the trench. This volcanic chain would be anomalous if it were related to the subduction of the Cocos plate off southern Mexico. In 1979, as part of the International Phase of Ocean Drilling (IPOD) of the Deep Sea Drilling Project, Legs 66 and 67 respectively explored the northern (off Acapulco, Mexico) and southern (off San Jose, Guatemala) section of the Mid America Trench<sup>3-6</sup> (Fig. 1). Since then short topographical surveys of the trench have been carried out by RV *Jean Charcot*<sup>8</sup> using the sea-beam technique<sup>7</sup>. We report here that both sets of data, together with UTMSI multichannel seismic profiles<sup>10,11</sup> can be used to reconstruct the processes of subduction along the Mid America Trench.

Leg 67 (refs 3, 4), in the southern section seems to include the first occasion that a drill-hole in an oceanic trench reached the oceanic basement. The sharp contrast between the Upper Cretaceous sequence at the base of the continental slope and the subducting Lower Miocene Cocos plate beneath it suggests that in the region of Leg 67, subduction is not accompanied by accretion. Data from Leg 66 (refs 4, 5) in the Northern section of the trench show an upslope increasing age of turbidites on the continental wall (Pleistocene up to Middle Miocene) indicating accretion. In the absence of more representative drilling data from the northern and southern sections, however, the results of Leg 67 could be explained by the slumping of a large block of Cretaceous material and those of Leg 66 by the trapping of coarse sediments in suspended ponded basins.

The results of the sea-beam surveys (Fig. 2a, b) confirm the general features of the Mid America Trench; they also give new data that clarify the mechanism of subduction in the northern and southern sections. The most striking feature of the data is that the Cocos plate is marked, in the neighbourhood of the trench, by elongated ponds and swells trending  $130^{\circ}$ - $140^{\circ}$  N, that are clearly oblique to the mean  $110^{\circ}$ - $120^{\circ}$  N direction of the trench (Fig. 2a, b). This horst and graben pattern, already known from previous seismic records<sup>10,11</sup>, is thus not as parallel



Fig. 1 Tectonic framework of the Mid America Trench.



to the trench axis as was assumed. In the Leg 66 area, at the mouth of the very active Ometepe canyon<sup>11</sup>, the Cocos pattern disappears towards the trench under a thick pile of turbidites (Figs 2b, d). In contrast, in the Leg 67 area, Cocos structures totally control the trench morphology. Indeed the trench consists of a succession of diamond shapes (corresponding to graben structures) and bottle-necks (corresponding to horsts) (Figs 2a, c). This is consistent with the drilling results at hole 500, just at the base of the continental slope, where a normal fault was suspected between Pleistocene trench sediments and Miocene chalk and where oceanic crust was reached 150 m above the same material in the trench<sup>3,4</sup>. Most probably hole 500 is just at the western edge of a Cocos horst. What about the origin of this oblique pattern? Is it an *en échelon* faulting due to oblique subduction or is it related to the Cocos plate internal grain? The latter solution is favoured by the clear parallelism observed between the horst and graben pattern and all known magnetic anomalies near the trench (refs 11, 12 and K. J. McMillen, unpublished data) (Fig. 2). Thus we consider that this structural pattern is controlled by inherited faults formed at the East Pacific Rise. Indeed it has been shown<sup>13,14</sup> that the genesis of oceanic crust along the East Pacific Rise is accompanied by dense normal faulting and fracturing, parallel with the spreading axis. These faults become inactive ~10 km off the rise<sup>13,14</sup>; however, they still exist as mechanical discontinuities. The bending of the Cocos plate in the subduction zone induces surficial tensional stress that preferentially reactivates these older fractures. The easier the reactivation, the smaller the angle (20–30°) between the mean direction of the trench (110°–120° N) and that of inherited fractures (130°–140° N). Such a reactivation process has already been proposed—with far less evidence—for the Nazca plate along the Peru–Chile trench<sup>15</sup>. The oblique subduction hypothesis can be rejected first, because the direction of convergence between the Cocos and the North America–Caribbean plates is well known<sup>16,17</sup>, about 30° N that is quite perpendicular to the trench along most of its length; and second, because off Salvador and Nicaragua where the trench trends 140° N and the subduction is thus oblique, our sea-beam survey shows the horst and graben pattern parallel with the trench.

The sea-beam map shows that in the Leg 67 area (Fig. 2a), the Cocos structures reach the base of the continental wall without disturbing it. The main thrust fault of the subduction zone is apparently between the continental slope, where Upper Cretaceous sediments were drilled in hole 494 and a Cocos horst where the Lower Miocene oceanic crust was reached in hole 500. Thus, off Guatemala, neither the drilling results nor the sea-beam surveys indicate that a classical accretionary prism<sup>2</sup>

has developed. The presence at the base of the continental slope of such a sequence involving Upper Cretaceous and Tertiary in good stratigraphical order still needs explanation. It could be part of a large block slumped down the slope in recent times; however, sea-beam data (Fig. 2a) indicate that the base of the innerwall is perfectly rectilinear and that no scar is present. Anyway, the Upper Cretaceous sequence could belong to a block slumped in pre-Pliocene or Miocene times: that would mean that no accretion has occurred at least since then. Finally, the whole sequence of hole 494 could be considered as *in situ* implying no accretion for more than 70 Myr or that tectonic erosion removed younger accreted terranes. Only new drilling will eventually solve the problem. In the Leg 66 area (Fig. 2b, d) the infilling of the trench means that the contact itself between Cocos structures and the innerwall is not visible. However, the sea-beam map shows a more or less continuous ridge in the middle of the trench. Such a central crest was first described off Costa Rica, at the very end of the trench, from submersible observations<sup>18</sup>. Off Southern Mexico the ridge has a maximum relative height of 100 m and is flanked on its seaward side by an elongated depression in which anastomosed channels of the Ometepe deep-sea fan seem to converge. Central ridge and channel define the limit between a relatively smooth outer trench and an undulated and uplifted inner trench. Sea-beam data confirm previous interpretations from UTMSI multichannel seismic reflection lines crossing the same area<sup>11</sup>. Thus the central ridge appears to be located above the most recent emergence of the Benioff plane. In the inner trench, turbiditic reflectors are gently folded; in particular, south-east of the Ometepe canyon mouth both reflection data and sea-beam maps show the inner trench as a synformal depression (Figs 2b, d). Upslope, on the continental wall, the same ridge-and-basin morphology is present but shows far more contrast and is mature. Site 488, drilled on the first ridge at the base of the innerslope, reached tectonized turbidites 400–500 m higher than the present-day trench turbidites<sup>5</sup>. Finally, although no tectonic repetition has been confirmed by Leg 66 drillings, the data strongly suggest that trench sediments are being accreted to the Mexican margin off Acapulco<sup>5,6,11</sup>.

Regarding the Cocos plate sea mounts related to off-axis volcanism are locally abundant and prevent development of a regular tectonic pattern. One of these sea mounts already mapped at 15°27'N–98°31'W off Mexico<sup>11</sup> is revealed by sea-beam bathymetry as a nice collapse-pit volcano about 6.5 km in diameter (Fig. 3a). Other sea mounts are smaller regular cones belonging to the Cocos Ridge off Costa Rica and entering the subduction zone without apparent deformation (Fig. 3b).

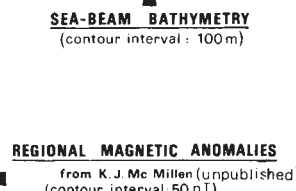
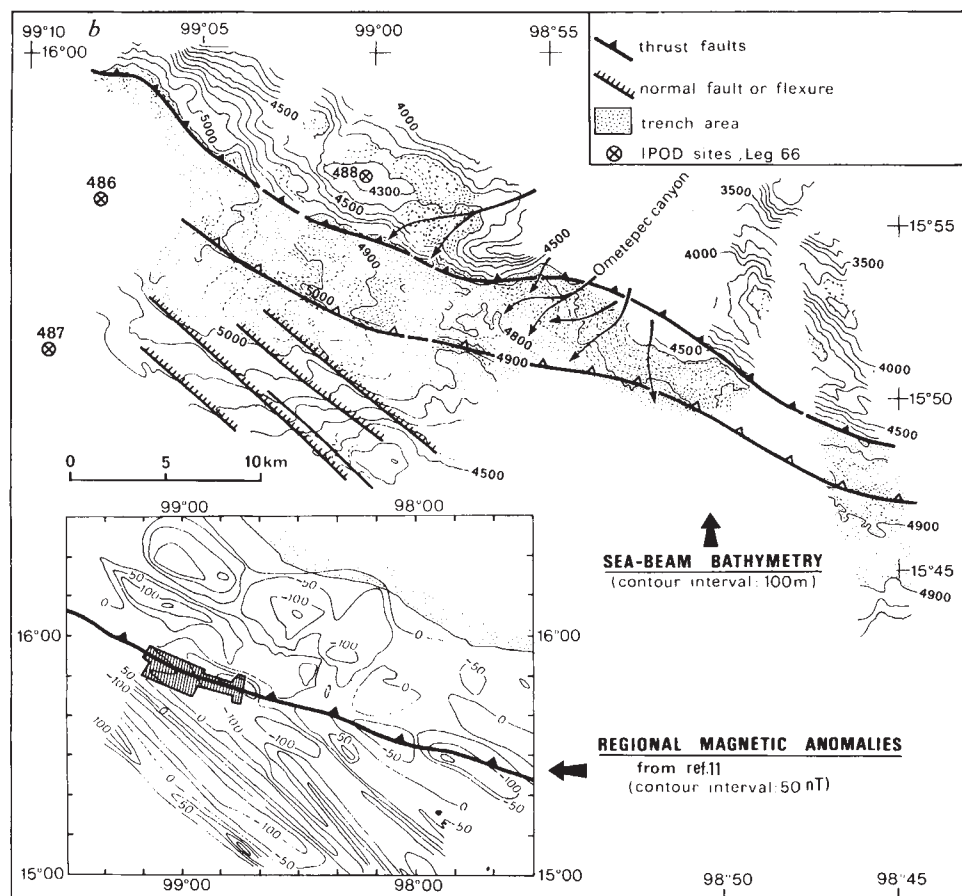
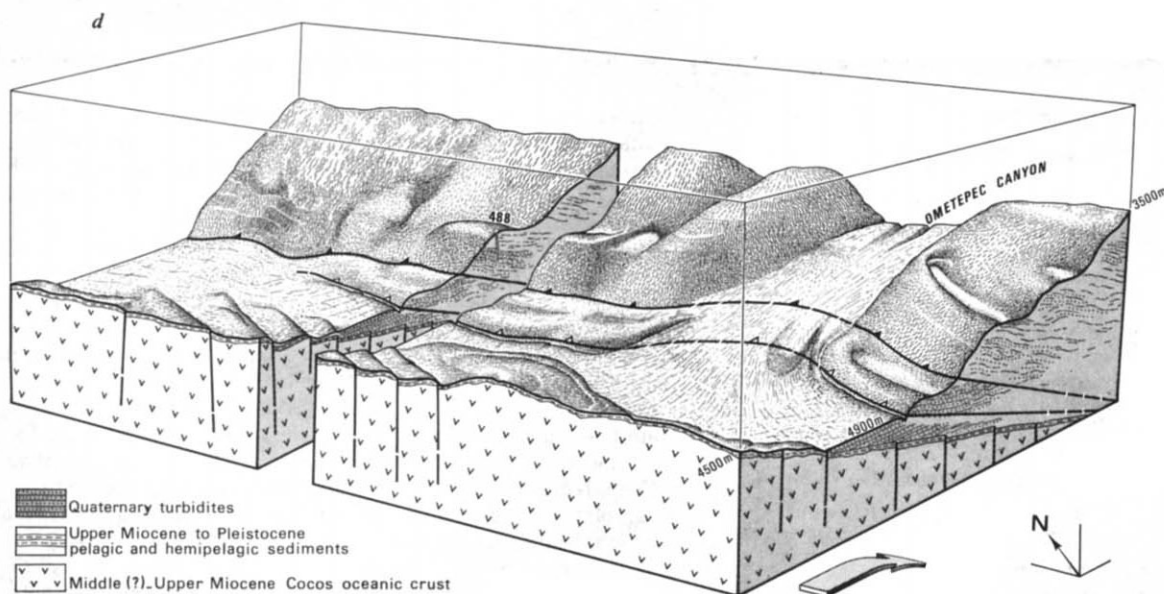


Figure 1 is a block diagram showing a cross-section of a mid-ocean ridge. The ridge is labeled "MID AMERICA TRENCH" at the top. The ridge axis is labeled "NEOGENE PALEOGENE U. CRETACEOUS". The ridge is flanked by "SEAWARD" and "LANDWARD" slopes. The ridge axis is labeled "499" and "494". The ridge axis is labeled "5000m" and "6000m". The ridge axis is labeled "1 km" and "2.5 km". The ridge axis is labeled "N" and "S". The ridge axis is labeled "SEAWARD" and "LANDWARD".



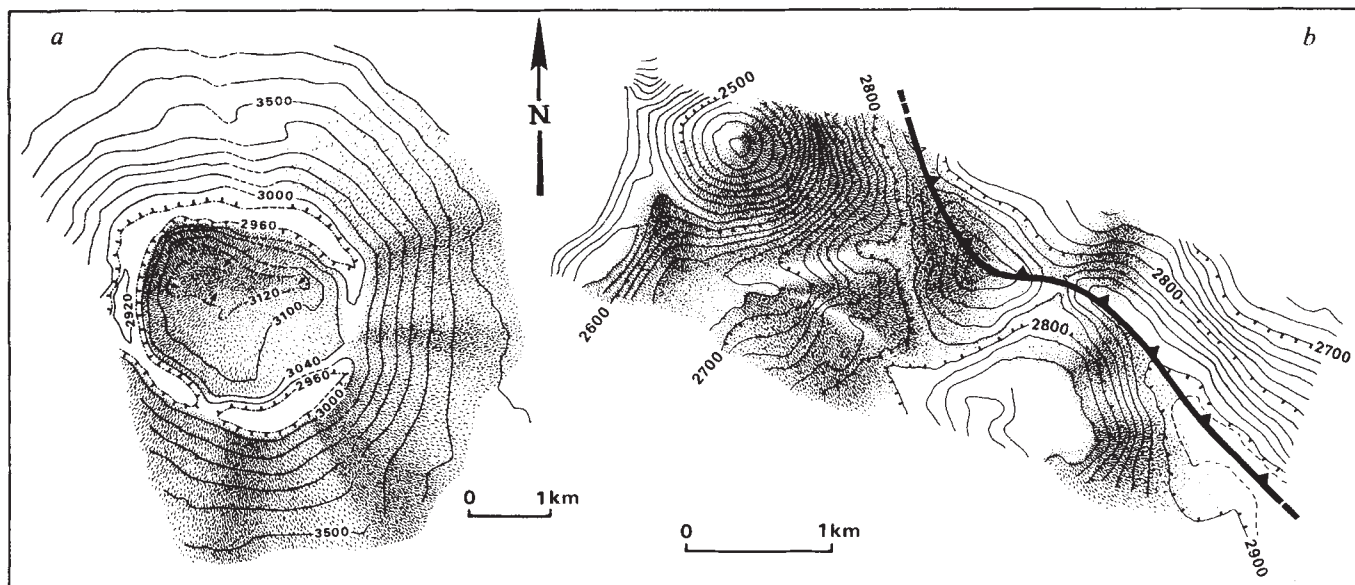


**Fig. 2** Sea-beam map of *a*, the Leg 67 area and *b*, the Leg 66 area. (The original 10-m contour interval has been condensed to 100 m.) Schematic diagram of *c*, the Leg 67 area (after ref. 9) and *d*, the Leg 66 area.



transport to the trench of the clastics supplied by wide mountainous areas in the background (Sierra Madre del Sur); to the south, off Central America, the fluvial input is far less important and most terrigenous material is trapped in the wide shelf basin. We know these two kinds of active margin belong to distinct plates (that is, the North America and the Caribbean plates). The boundary between both margins is located in the Gulf of Tehuantepec<sup>2,20</sup> that is exactly in the prolongation of the Polochic-Motagua fault system (Fig. 1). Thus, the northern and southern margins of the Mid America Trench directly reflects the geodynamic evolution of their own plate. For example, the

Mexican margin has experienced in Lower Tertiary times tectonic truncation and erosion that scraped off all Mesozoic accreted terranes as well as part of the older basement<sup>11,12,22,23</sup>. In contrast, along the Central America margin a fore-arc basin has developed at least since the Palaeocene; moreover, a belt of Mesozoic ophiolites is still preserved on the margin<sup>2,10</sup>. Regarding the present, tensional stress is responsible for the genesis of all known graben (Guatemala City, Nicaragua, and so on) south of the Polochic-Motagua fracture zone, while north of this boundary compressive deformations are occurring<sup>24</sup>. We conclude therefore that not only simple parameters (such as



**Fig. 3** Collapse-pit volcano off Mexico (a) and sea mounts on the Cocos ridge (b). See location on Fig. 1. Contour interval, 100 m on a; 20 m on b.

convergence rate and trench fill) account for the variation of the subduction process but also the properties (such as structure and kinematics) of the overriding plate. The Mid America Trench is

a useful area for investigating the problem and for determining how far the upper plate governs the dynamic of the subduction process.

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1. Molnar, P. & Sykes, L. R. *Bull. geol. Soc. Am.* **80**, 1639–1684 (1969).
2. Seely, D. R. *Am. Ass. petrol. Geol. Mem.* no. 29 (1979).
3. Aubouin, J. et al. *C. r. heb. Séanc. Acad. Sci. Paris* **289**, 1215–1220 (1979).
4. Huene, R. von et al. *Bull. geol. Soc. Am.* **91**, 421–432 (1980).
5. Moore, J. C. et al. *Nature* **281**, 638–642 (1979).
6. Moore, J. C. et al. *Geotimes* **24**, 20–22 (1979).
7. Renard, V. & Allenou, J. P. *Revue Hydrograph. Int.* **56**, 35–71 (1979).
8. Renard, V., Aubouin, J., Lonsdale, P. & Stephan, J. F. *C. r. heb. Séanc. Acad. Sci., Paris* **D291**, 137–142 (1980).
9. Aubouin, J. et al. *26th int. geol. Congr., Paris* (1980); *Oceanologica Acta, Suppl.* **4**, 225–232 (1981).
10. Ladd, J. W. et al. *Proc. Int. Sym. Guatemala Earthquake and Reconstruction, Guatemala City* (1978).

11. Shipley, T. H., McMillen, K. J., Watkins, J. S., Sandoval-Ochoa, J. H. & Worzel, J. L. *Mar. Geol.* **35**, 65–82 (1980).
12. Karig, D. E., Cardwell, R. L., Moore, G. F. & Moore, D. G. *Bull. geol. Soc. Am.* **89**, 265–276 (1978).
13. Cyamex Scientific Team *Mar. geophys. Res.* **4**, 345–379 (1981).
14. Rangin, C. & Francheteau, J. *Oceanologica Acta* (in the press).
15. Coulbourn, W. T. & Moberly, R. *Can. J. Earth Sci.* **14** (1), 102–116 (1977).
16. Dean, B. W. & Drake, C. L. *J. geol.* **86**, 111–128 (1978).
17. Minster, J. B. & Jordan, T. H. *J. geophys. Res.* **83**, 5331–5354 (1978).
18. Heezen, B. C. & Rawson, M. *Science* **196**, 423–425 (1977).
19. Truchan, M. & Larson, R. *Earth planet. Sci. Lett.* **17**, 426–432 (1973).
20. Couch, R. & Woodcock, S. J. *geophys. Res.* **86**, 1829–1840 (1981).
21. Ross, D. A. *Bull. geol. Soc. Am.* **82**, 303–322 (1971).
22. Aubouin, J. *7ème conf. géol. Caraïbe, Pointe-à-Pitre*, 41–59 (1974).
23. Bellon, H., Maury, R. & Stephan, J. F. *Init. Rep. DSDP* **66** (in the press).
24. Plakfer, G. *Science* **193**, 1201–1208 (1976).

## Peralkaline volcanicity on the Eurasia Basin margin

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The Kap Washington Group of post-Palaeozoic explosive volcanic rocks was discovered in 1969 on the north coast of Greenland. Although there have been uncertainties regarding their age and chemical character, they have featured prominently in geotectonic reconstructions of the Arctic regions—in recent interpretations as products of the Yermak hot spot, generated on the Nansen spreading axis during the opening of the Eurasia Basin. We present here new evidence which confirms the volcanicity as end-Cretaceous in age and of peralkaline type. We show that a direct connection with the Yermak hot spot is improbable and infer that the volcanic rocks were generated in a continental extensional rift environment before the break-up of the Laurasian plate in the Arctic. Their age helps to constrain the timing of this poorly understood event.

Aeromagnetic surveys conducted in the past decade<sup>1–3</sup> have established that the Eurasia Basin developed in Cenozoic time by ocean-floor spreading on the Nansen (Gakkel) Ridge, which detached an elongated continental fragment (the Lomonosov Ridge) from the Barents margin of the Eurasian plate (Fig. 1). There are problems concerning both the geometry and chronology of this phase of spreading. The geometrical problems arise from the uncertainties which still exist concerning the origin of the Amerasia Basin and Alpha Ridge<sup>2,4</sup> and also the constraints imposed by the limited displacement permissible on the Nares Strait line<sup>5</sup> on the geometry of opening of the Labrador Sea and Baffin Bay. Although recent magnetic anomaly superposition studies<sup>1–4,6–9</sup> have shed much light on the displacement patterns of the individual ocean basins around Greenland, it is clear that rigid-plate models do not lead to a complete geometrical solution and that within-plate strains must be important. Such strains are evidenced by the belt of Tertiary deformation which extends from the Canadian Arctic Archipelago (Eurekan Orogen<sup>10,11</sup>) across northern Greenland<sup>12,13</sup> to Svalbard (West Spitsbergen Orogen<sup>14</sup>).

The main chronological problem posed by the evidence from the Eurasia Basin is the time at which spreading was initiated. In dating magnetic anomalies and in relating on-land geological evidence to the history of the oceans, it is essential to use a recent polarity time scale. We adopt that of Hailwood *et al.*<sup>15</sup>, noting that the commonly used Heirtzler *et al.*<sup>16</sup> scale assigns ages to magnetic epochs in the late Cretaceous and early Tertiary which are too high by ~10%. The oldest ocean-floor anomaly recognized in the Eurasia Basin is 24 (52 Myr, early Eocene) and a broad negative anomaly exists over the 50–100 km wide zone between it and the Lomonosov and Barents continental slopes, in which there is space to accommodate anomalies 25–28 (ref.



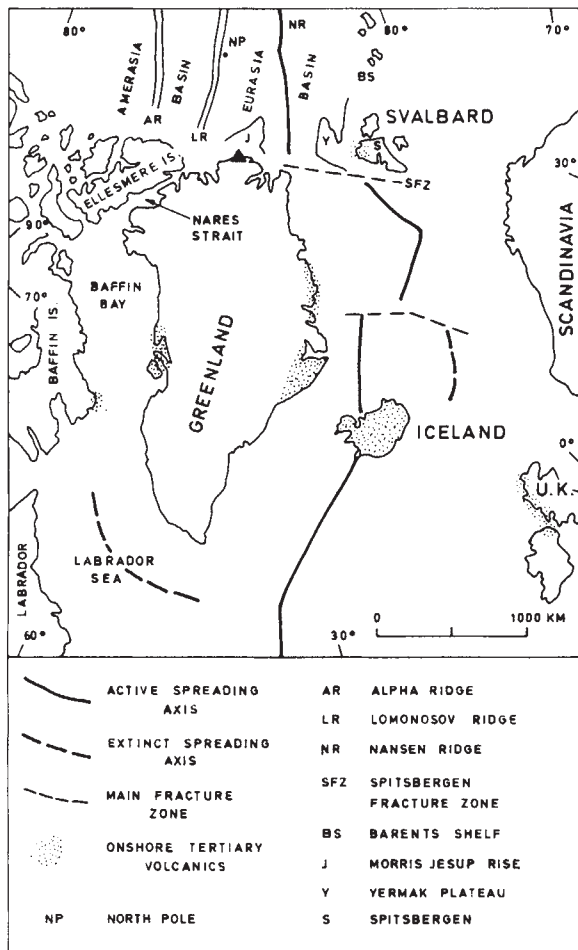


Fig. 1 Index map of the North Atlantic region. ▲, The location of the Kap Washington Group of volcanics in northern Greenland.

2). The crust flooring this zone could thus represent subsided continental crust, oceanic crust generated during a pre-anomaly 24 quiet period, or oceanic crust generated during the time of anomalies 25–28 (55–63 Myr, Palaeocene), whose magnetic expression was subsequently erased. This uncertainty should be seen in the light of the recently suggested history of the Amerasia Basin where seafloor spreading anomalies 34–19 (Cretaceous to mid-Eocene) are deemed identifiable between the Lomonosov Ridge and Alpha Ridge<sup>4</sup>.

Notable features in the present context are the aseismic submarine plateaus off northern Greenland (Morris Jesup Rise) and Svalbard (Yermak Plateau). These are thought to be constructional features produced by enhanced basaltic discharge from a Yermak hot spot situated at the junction of the Nansen Ridge and the Spitsbergen Fracture Zone<sup>3</sup> (Fig. 1). High-amplitude magnetic anomalies are associated with these plateaus, and they are bracketed by ocean-floor anomalies 13–18 (35–40 Myr, late Oligocene–early Eocene). This is thought to represent the period of maximum hot spot activity after which the subsiding plateaus were rifted apart by continued spreading. It has been proposed<sup>3</sup> that the volcanic province on the north coast of Greenland (Kap Washington Group<sup>12,17</sup>) is an early manifestation of this hot spot activity. If this were the case, the age of the volcanic rocks would provide an indication of the time of initiation of the hot spot volcanism, and thus of the onset of spreading in the Eurasia Basin. However, our data on the age and chemical character of the Kap Washington Group volcanics reported here demonstrate that the eruptives represent the products of peralkaline rift volcanism that predates the spreading history of the Eurasia Basin and thus the eruptives are unlikely to be directly associated with the Yermak hot spot.

The Kap Washington Group volcanic rocks outcrop at about 83°30'N on the north coast of Greenland at the edge of the

North Greenland fold belt, where they have a chronological position postdating the mid-Palaeozoic diastrophism of the fold belt but predating its Tertiary reactivation. Limited field observations in 1969<sup>12,18</sup> have been supplemented by field work in 1979<sup>19</sup> and 1980<sup>20</sup>. The outcrop of the volcanic rocks is limited to ~150 km<sup>2</sup> (Fig. 2), but considerable offshore extension may be indicated by the Morris Jesup Rise magnetic anomaly<sup>3,21</sup>, although the nature of the rocks responsible for the anomaly is not known.

The extrusive volcanic rocks form southeasterly inclined sequences; they are non-metamorphic but locally show shearing and crushing and in places cleavage. The thickness of the exposed volcanic sequence is at least 5 km. It is composed of prominent pyroclastic rocks, including coarse breccias, finely bedded airfall tuffs, accretionary lapilli tuffs and welded ash-flow tuffs, as well as both basic and acidic lavas. Some intrusive rock types are present, mainly felsic dykes and small plugs. The upper boundary of the volcanic sequence is a thrust contact with Lower Palaeozoic metasediments of the fold belt; thrusts also disturb the volcanic succession and bring in wedges of Carboniferous and Permian strata of the Wandel Sea Basin (Fig. 2).

Hitherto unpublished chemical data for material collected in 1969<sup>18</sup> and new petrographical data for recently collected material<sup>20</sup> demonstrate that the Kap Washington Group volcanics represent a peralkaline suite. The 1969 material is mainly rhyolitic and is difficult to characterize chemically; on a silica/alkali plot the rocks fall, not unexpectedly, in the sub-alkaline field. The single available analysis of a basic flow is thoroughly alkaline. The 10 analyses of rhyolitic material (SiO<sub>2</sub> range 66–75%) show appreciable alteration of major element composition and there is a marked variation in the Na<sub>2</sub>O/K<sub>2</sub>O ratio (total alkalis ~8.5%). CaO varies from 0.02 to 1.83 and Al<sub>2</sub>O<sub>3</sub> is between 10 and 18%. However, two samples show normative acmite—a clear indication of peralkalinity—and the trace element composition, particularly with respect to the relatively high values of Nb, Zr and Y, conform to those values normal for peralkaline rocks<sup>22</sup>. Petrographical results indicate that basalts, riebeckite trachytes and riebeckite comendites or pantellerites are the prominent lava types. Some basalts have a strongly developed flow texture and may overlap into the compositional field of hawaiites. The trachytes contain anorthoclase, ill-formed riebeckite and quartz in the flow textured groundmass. The more siliceous lavas have a variable riebeckite content and include flow banded and spherulitic varieties. The presence of the soda amphibole riebeckite establishes the peralkaline character of the Kap Washington Group.

The Kap Washington Group volcanic rocks conformably overlie black shales and sandstones which contain fragmentary plant debris. While no microfossils have been isolated from the sediments, a few angiosperm leaf fragments indicate a mid-Cretaceous or younger age. The available angiosperm leaf fragments are not determinable at either genus or species level. One small, nearly entire leaf specimen is a very primitive morphological type appearing in the Lower Cretaceous (Albian) floras of the eastern USA and Portugal (*Proteaphyllum* Fontaine and similar form genera). Another is a leaf fragment of an advanced pinnate angiospermous leaf type with percurrent nervils appearing in the Upper Cretaceous and continuing through the Tertiary. The primitive leaf type makes a Cretaceous age probable for the shale in question. The advanced leaf type does not permit an age older than mid-Cretaceous. However, with the very limited material available this can only be a tentative age.

Other black shales are interbedded with the pyroclastic rocks some 3 km above the base of the volcanics and several samples have been subjected to palynological investigation. The microfossils recovered are generally in a very poor state of preservation and few can be identified. Bisaccates, smooth-walled triradial spores and some distinctive angiosperm pollen grains, identified as species of *Aquilapollenites*, *Mancicorpus* and *Triprojectus*, have been recorded, but all other identifications are questionable. Fortunately, the named genera have restricted

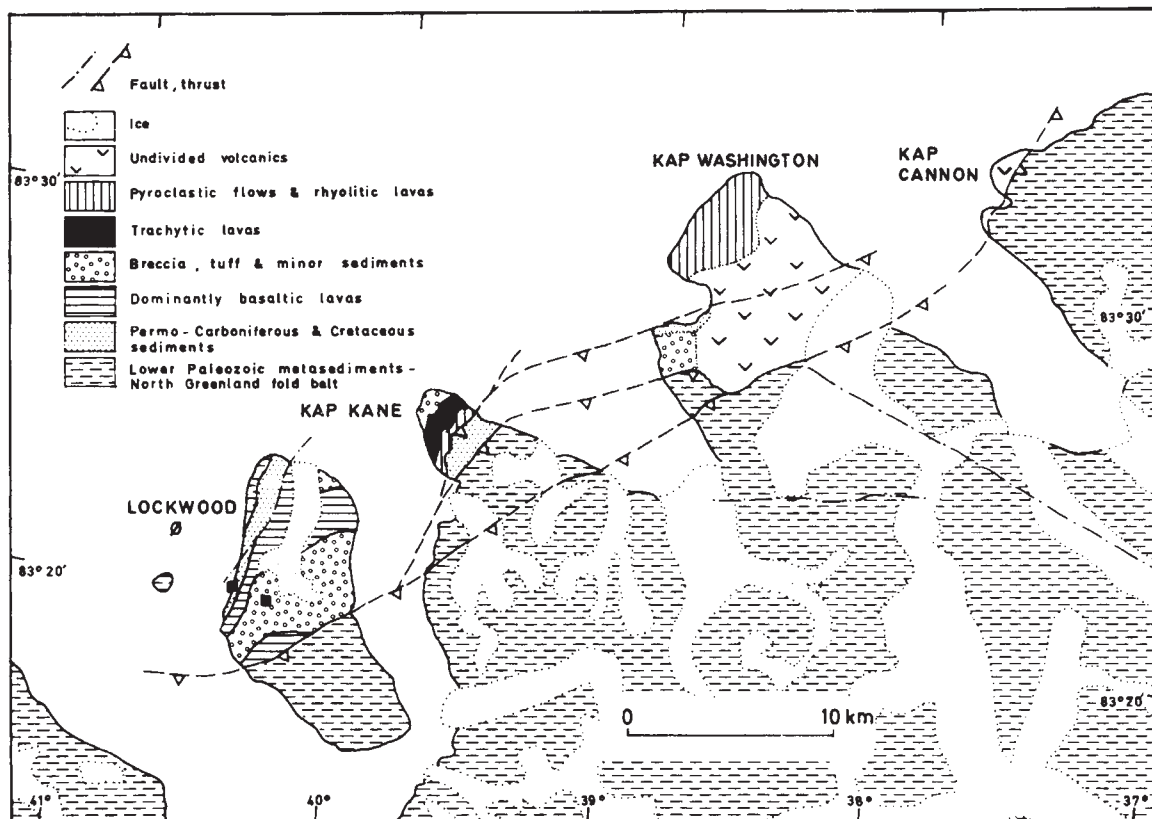


Fig. 2 Geological map of the total outcrop of Kap Washington Group volcanics. ■, Localities of the fossiliferous shales.

stratigraphical ranges and a late Cretaceous Campanian or Maastrichtian age is indicated; that is 75–65 Myr on the stratigraphical time scale. The upper limit is in close agreement with an earlier Rb–Sr whole-rock age of  $63 \pm 2$  Myr on five rhyolitic samples<sup>23</sup>, a result which had previously been regarded as unreliable on technical grounds. An age for the volcanicity at, or just before, the Cretaceous–Tertiary boundary is adopted here.

The characteristic tectonic environment of peralkaline volcanism is one of crustal extension and attenuation, so that their most abundant development is in association with regional doming and rifting<sup>24</sup>. Evidence of crustal extension immediately preceding the extrusion of the Kap Washington volcanics is provided by alkali dolerite dykes and sills<sup>13</sup>. These cut the early Cretaceous and Carboniferous–Permian sediments, as well as older rocks of the North Greenland fold belt, but fail to penetrate the Kap Washington Group volcanic rocks<sup>25</sup>. These dykes, like the volcanics, show alteration and some shearing due to Tertiary tectonism. K/Ar whole-rock determinations show a range of apparent ages, although the most reliable of these away from areas of known Tertiary tectonism lie between 82 and 66 Myr (ref. 26). The dyke swarms are regarded as late Cretaceous in age.

The composition of the Kap Washington volcanics illustrates that the tectonic regime in the Barents Shelf–Greenland region in late Cretaceous time was extensional, as indeed it was in the entire region from the Sverdrup Basin of Arctic Canada through northern Greenland to the northern Barents Shelf. The palaeontological dating of the Kap Washington Group reported here places the beginning of the active spreading in the Eurasia Basin as probably no earlier than the Cretaceous–Tertiary boundary (perhaps as early as 75 Myr). The various reconstructions of the Barents Shelf margin before the seafloor spreading<sup>1,3,4,9,13</sup> place Svalbard close to northern Greenland and it has been suggested that the Kap Washington volcanics and even the earlier basic dykes are manifestations of the Yermak hot spot<sup>3</sup>. On the grounds of both their age and composition, we would not relate the Kap Washington Group or the basic dykes directly to the Yermak hot spot; the dykes and

volcanics are late and end-Cretaceous in age, respectively, while the first manifestation of hot spot activity in the ocean floor is at anomaly 18 time (40 Myr, late Eocene). The geographical association of the alkaline rift magmatism and later hot spot activity may well have petrogenetic significance, but this problem must await petrological studies of the Kap Washington volcanics and also sampling of the hot spot rocks from the submarine plateaus. Further consideration of Cretaceous–Tertiary magmatic and tectonic events in northern Greenland and their relationships to the history of adjacent ocean basins is presented elsewhere<sup>13</sup>.

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- Phillips, J. D. *et al.* *Bull. geol. Soc. Am.* (in the press).
- Vogt, P. R. *et al.* *J. geophys. Res.* **84**, 1071–1089 (1979).
- Feden, R. H. *et al.* *Earth planet. Sci. Lett.* **44**, 18–38 (1979).
- Taylor, P. T. *et al.* *J. geophys. Res.* **86**, 6323–6333 (1981).
- Christie, R. L. *et al.* *Nature* **291**, 478–480 (1981).
- Kristoffersen, Y. & Talwani, M. *Bull. geol. Soc. Am.* **88**, 1037–1049 (1977).
- Talwani, M. & Eldholm, O. *Bull. geol. Soc. Am.* **88**, 969–999 (1977).
- Srivastava, S. P. *Geophys. J. R. astr. Soc.* **52**, 313–357 (1978).
- Kovacs, L. C. in *Nares Strait: a Central Conflict in Plate Tectonic Studies of the Arctic* (eds Dawes, P. R. & Kerr, J. W.) (Meddr Grønland, Geoscience, in the press).
- Thorsteinsson, R. & Tozer, E. T. *Geol. Surv. Can., Econ. Geol. Rep.* **1**, 549–590 (1970).
- Trettin, H. P. *Geol. Ass. Can. Spec. Pap.* **11**, 83–179 (1972).
- Dawes, P. R. & Soper, N. J. *Mem. Am. Ass. petrol. Geol.* **19**, 117–134 (1973).
- Soper, N. J. *et al.* in *Nares Strait: a Central Conflict in Plate Tectonic Studies of the Arctic* (eds Dawes, P. R. & Kerr, J. W.) (Meddr Grønland, Geoscience, in the press).
- Harland, W. B. & Horsfield, W. T. *Geol. Soc. Lond. Spec. Publ.* **4**, 745–755 (1974).
- Hailwood, E. A. *et al.* *Init. Rep. DSDP* **48**, 1119–1141 (1979).
- Heirtzler, J. R. *et al.* *J. geophys. Res.* **73**, 2119–2136 (1968).
- Dawes, P. R. in *Geology of Greenland* (eds Escher, A. & Watt, W. S.) 248–303 (Geological Survey of Greenland, 1976).
- Dawes, P. R. & Soper, N. J. *Rapp. Grønlands geol. Unders.* **28**, 9–15 (1970).
- Soper, N. J. *et al.* *Rapp. Grønlands geol. Unders.* **99**, 89–98 (1980).
- Brown, P. E. & Parsons, I. *Rapp. Grønlands geol. Unders.* (in the press).
- Dawes, P. R. in *Continental Drift, Sea Floor Spreading and Plate Tectonics: Implications to the Earth Sciences* (eds Tarling, D. H. & Runcorn, S. K.) **2**, 917–939 (Academic, London, 1973).
- MacDonald, R. & Bailey, D. K. *U.S. geol. Surv. Prof. Pap.* 440-N-1 (1973).
- Larsen, O. *et al.* *Rapp. Grønlands geol. Unders.* **90**, 115–119 (1978).
- MacDonald, R. *Bull. volcan.* **34**, 575–593 (1974).
- Higgins, A. K. *et al.* *Rapp. Grønlands geol. Unders.* (in the press).
- Dawes, P. R. & Soper, N. J. *Rapp. Grønlands geol. Unders.* **93**, (1979).



# Migration of late Cenozoic volcanism in the South Island of New Zealand and the Campbell Plateau

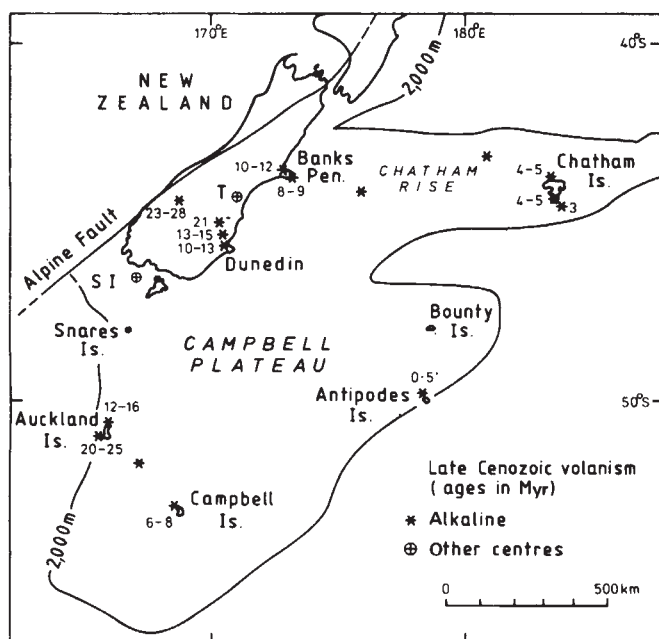
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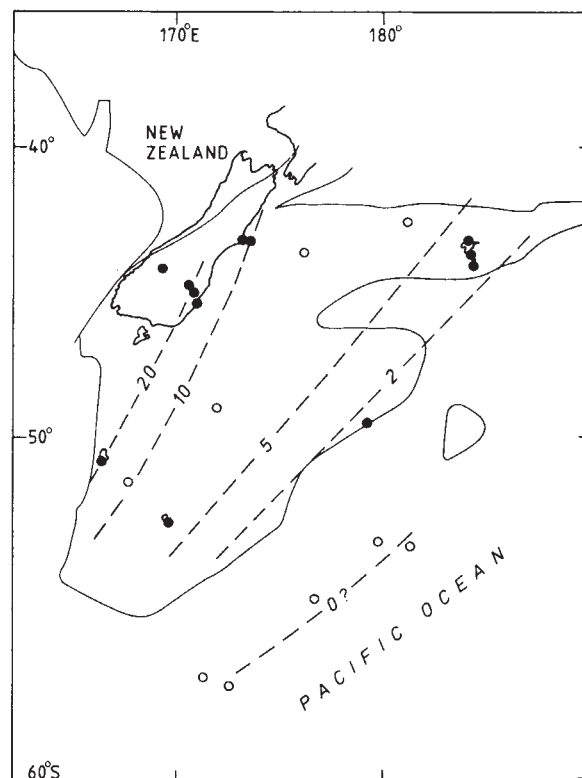
In late Cenozoic volcanics of eastern Australia, Wellman and McDougall<sup>1</sup> have shown a remarkable southward migration pattern of major shield volcanoes of alkaline olivine basalt association which they relate to movement of the continental crust over a fixed mantle hot-spot. Contemporaneous tholeiite lava field provinces do not show a similar pattern. Following their general selection criteria<sup>1</sup>, I believe a similar situation emerges for New Zealand examples. In the South Island of New Zealand and the adjacent Campbell Plateau and Chatham Rise are several late Cenozoic volcanic centres that rest on a peneplain in Mesozoic and Palaeozoic granites and metamorphic rocks<sup>2,3</sup>, which is partly mantled by late Cretaceous to mid-Tertiary shallow marine sediments<sup>4,5</sup>. These major centres of late Cenozoic alkalic volcanism show an eastward decrease in age from ~28 Myr to ~0.5 Myr, presumably reflecting movement of a linear mantle source with respect to the Pacific Plate.

The volcanic centres are widely scattered and three volcanic associations are present: (1) Several major shield volcanoes of alkaline olivine basalt association, for example, Banks Peninsula, Otago Peninsula, Auckland and Campbell and Chatham Islands. (2) Very minor and restricted tholeiite, for example, Timaru Basalt. (3) A calc-alkaline andesite volcano at Solander Island (probably related to active eastward subduction of the Indian plate below Fiordland). Brief geological notes for these volcanoes are compiled in Table 1 and age distributions shown in Fig. 1. In defining the age trend, only data from major alkaline olivine basalt centres are used, that is, the first group described above.

Although the data are sparse, there is an eastward decrease in age for the volcanic centres, and although there are no marked chains of volcanoes, there seems to be a predisposition for



**Fig. 1** Age data for centres of alkaline volcanism of mid-late Tertiary age in southern New Zealand and Campbell Plateau. SI, Solander Island; T, Timaru Basalt.



**Fig. 2** Approximate locus of alkaline volcanism during late Tertiary on the basis of age data summarized in Fig. 1 from major volcanic centres dated (●). Possible submarine volcanic edifices, of similar age and type (○)<sup>4,5,29</sup> but undated.

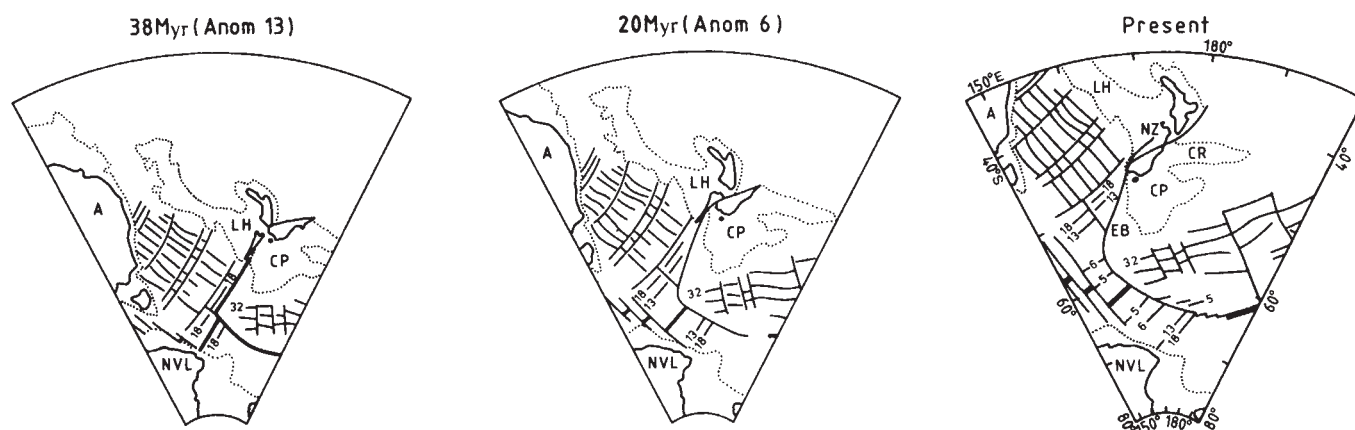
volcanism to occur, at any one time, within a geographically restricted belt. The centres form neither arcs (as, for example, in the case of calc-alkalic volcanism) nor linear chains that arise from 'hot-spot' volcanicity. Using these age data Fig. 2 shows approximate isochrons which form a fan-shaped set beginning at 20 Myr ago, crossing the South Island in an NNE/SSW direction and rotating to a present NE/SW position close to the Campbell Plateau continental slope. This migration parallels the late Cenozoic movement of the New Zealand continental crust with respect to the active mid-oceanic spreading centre in the southwest Pacific (that is, an anticlockwise rotation of 60° about a pole approximately 65° S, 140° E).

Thus the location of the alkaline volcanism seems to be directly related to the passage of a mantle source below. At the surface there is evidence that the volcanism is locally controlled by major tectonic features: for example, Miocene NNE/SSW reverse faulting in east Otago<sup>6</sup> and recent NE/SW faults in the Antipodes Island and Campbell Plateau margin<sup>7</sup>. It is possible that the locus and style of faulting are themselves an expression of the passage of the hot mantle source below. A more speculative suggestion is that broad regional uplifts in the Oligocene-Miocene in Otago<sup>8</sup> and more recently on the Auckland/Campbell Island platforms<sup>4</sup> might also reflect this process. The mantle source in this case cannot be regarded as a single 'hot spot', similar to, for example, that which produced the Hawaiian volcanic island chain, or the slightly more diffuse source beneath the east Australian Cenozoic volcanoes. In the New Zealand-Campbell Plateau example, the mantle source responsible for the Cenozoic volcanism forms a relatively narrow (~100–200 km) linear belt, and is 1,000–1,500 km long throughout its course beneath southern New Zealand and the Campbell Plateau region. The movement of the mantle source corresponds closely to known late Cenozoic motion of the Pacific Plate with respect to the mantle. Hatherton<sup>9</sup> indicated a similar migration pattern for the late Cenozoic-Quaternary calc-alkaline volcanic belts of the North Island of New Zealand formed at the Indian/Pacific Plate boundary. Using the poles and movement

**Table 1** Cenozoic volcanic centres of the south-west Pacific region

| Area                                         | Volcanic association          | Rock type and trends               | Age (Myr)         | Ref.   |
|----------------------------------------------|-------------------------------|------------------------------------|-------------------|--------|
| South Island, New Zealand                    |                               |                                    |                   |        |
| Banks Peninsula                              |                               |                                    |                   |        |
| Akaroa centre                                | Alkaline, shield volcano      | Basalt-hawaiite-trachyte           | 8-9               |        |
| Lyttleton centre (i)                         | Alkaline                      | Hawaiite                           | 10-12             | 11-13  |
|                                              | Alkaline                      | Basalt                             | 5-8               |        |
| (ii)                                         | Alkaline, dyke swarm          | Basalt, lamprophyre                | 20-28             | *      |
| Wanaka                                       |                               |                                    |                   |        |
| Dunedin                                      |                               |                                    |                   |        |
| Otago Peninsula                              | Alkaline shield volcano       | Basalt-hawaiite-trachyte-phonolite | 10-13             | 14-17  |
| Waipiata province                            | Alkaline                      | Basalt, basanite                   | 12-15, 21         | 6*     |
| Timaru                                       | Tholeiitic, flood basalt      | Basalt                             | 2.5               | 18     |
| Solander Island                              | Calc-alkaline volcano         | Andesite                           | Pleistocene?      | 19     |
|                                              | Pacific/Indian Plate boundary |                                    |                   |        |
| Campbell Plateau-Chatham Rise, Pacific Ocean |                               |                                    |                   |        |
| Campbell Island                              | Alkaline, shield volcano      | Basalt-hawaiite-mugearite-trachyte | 6.5-11            | 3, 20  |
| Auckland Islands                             | Alkaline                      |                                    |                   |        |
| Ross Volcano                                 |                               | Basalt-mugearite-trachyte-rhyolite | 12-16             | 20-26  |
| Carnley Volcano                              |                               | Basalt-trachyte                    | 20-25             | *      |
| Antipodes Islands                            | Alkaline                      | Ankaramite, basalt                 | 0.2-0.5           | 7      |
| Chatham Islands                              |                               |                                    |                   |        |
| Northern centre                              | Alkaline                      | Limburgite                         | 35                |        |
|                                              | Alkaline, agglomerate vents   | Limburgite                         | 4-5               | 27, 28 |
| Pitt Island centre                           | Alkaline, agglomerate vents   | Limburgite-phonolite               | 2.6-5             |        |
| Chatham Rise                                 |                               | Basalt                             | Miocene-Pliocene? | 29     |

\* C.J.A., unpublished K-Ar data.

**Fig. 3** Present day seafloor magnetic anomaly pattern and reconstructions of the south-west Pacific region 20 Myr and 38 Myr ago from Figs 13, 14 and 15 of ref. 30. A, Australia; CP, Campbell Plateau; CR, Chatham Rise; EB, Emerald Basin; LH, Lord Howe Rise; NVL, North Victoria Land, Antarctica; NZ, New Zealand.

rates deduced from seafloor spreading data, one can show that the mantle source responsible for the alkalic volcanism of the southern New Zealand region must now lie to the south-west of the Campbell Plateau margin and in the southwestern Pacific Basin. In this way a presumed 'zero' isochron is shown in Fig. 2 and it is intriguing that this lies close to prominent submarine features interpreted by Summerhayes<sup>4</sup> as possible volcanic seamounts. However, these have never been sampled or dated.

Similarly, the earlier history of the mantle source can be deduced from known mid-Cenozoic plate motions. The oldest and westernmost alkaline igneous activity is in west Otago, where a late Oligocene basaltic and lamprophyre dyke swarm occurs. Thus a mantle source must have originated there, or immediately to the west and before the late Oligocene.

It is likely that the long linear mantle source is related to a process of major reorganization of the Indian-Pacific-Antarctic plate motions in the southern New Zealand region in early-Oligocene times (~38 Myr; Fig. 3), and particularly to the formation of the Moonlight Aulacogen<sup>8,10</sup> at the southwestern margin of the Pacific plate. The development of this aulacogen controlled Oligocene sedimentation in the Emerald and Solander Basins (at the western margin of the Campbell Plateau) and the on-land extension northward into the Waiau Basin and Moonlight Tectonic Zone up to the Alpine Fault. The dimensions of the mantle source inferred from the pattern of volcanic centres would certainly be compatible with a linear tensional

feature such as the Moonlight Aulacogen. The sub-crustal processes producing this aulacogen also possibly provided a mantle source for subsequent alkaline volcanism. However, immediately after this, a new phase of late Cenozoic seafloor spreading occurred in the southwestern Pacific (Fig. 3). Thus the continental crust of southern New Zealand and the Campbell Plateau began to move northwestwards over the mantle source and the subsequent alkaline volcanism is now expressed in a pattern of eastward migrating centres. The origin and generation of this new mantle source in relation to the early Cenozoic history is discussed in a new geophysical model of the region by Farrar, Adams and Dixon (in preparation).

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- Wellman, P. & McDougall, I. *Tectonophysics* **23**, 49 (1974).
- Fleming, C. A. *Jl. geol. Soc. Lond.* **125**, 125 (1969).
- Adams, C. J., Morris, P. A. & Beggs, J. M. *N.Z. J. geol. Geophys.* **22**, 679 (1979).
- Summerhayes, C. P. *Bull. N.Z. Dept. Sci. Ind. Res.* **190** (1969).
- Davey, F. J. *N.Z. J. geol. Geophys.* **20**, 719 (1977).
- McDougall, I. & Coombs, D. S. *N.Z. J. geol. Geophys.* **16**, 179 (1973).
- Cullen, D. J. *J. geophys. Res.* **74**, 4213 (1969).
- Carter, R. M. & Norris, R. J. *Earth planet. Sci. Lett.* **31**, 85 (1976).
- Hatherton, T. *N.Z. J. geol. Geophys.* **5**, 1204 (1967).
- Norris, R. J., Carter, R. M. & Turnbull, I. M. *J. geol. Soc. Lond.* **135**, 191 (1978).
- Liggitt, K. A. & Gregg, D. R. *N.Z. Dept. Sci. Ind. Res. Inf. Ser.* **51**, 9 (1965).
- Stipp, J. J. & McDougall, I. *N.Z. J. geol. Geophys.* **11**, 1239 (1968).
- Evans, A. L. *Geophys. J.R. astr. Soc.* **21**, 163 (1970).
- Benson, W. N. *Trans. R. Soc. N.Z.* **71**, 208 (1941).



15. Benson, W. N. *Trans. R. Soc. N.Z.* **72**, 85 (1942).
16. Benson, W. N. *Lex. Strat. Int.* **6**, 91 (1959).
17. Coombs, D. S. & Wilkinson, J. F. G. *J. Petrol.* **10**, 440 (1969).
18. Mathews, W. H. & Curtis, G. H. *Nature* **212**, 979 (1966).
19. Harrington, H. J. & Wood, B. L. *N.Z. J. geol. Geophys.* **1**, 419 (1958).
20. Oliver, R. L., Finlay, H. J. & Fleming, C. A. *N.Z. Dept Sci. Ind. Res. Cape Exp. Ser. Bull.* **3** (1950).
21. Speight, R. & Finlayson, A. M. *The Sub-Antarctic Islands of N.Z.* (ed. Chilton, C.) (1909).
22. Fleming, C. A. *Preliminary results of the Auckland Islands Expedition 1972-73* N.Z. Dept. Lands & Survey Rep. 3 (ed. Yaldwyn, J. C.) (1973).
23. Fleming, C. A. *Lex. Strat. Int.* **6**, 34 (1957).
24. Wright, J. B. *Trans. R. Soc. N.Z.* **5**, 71 (1967).
25. Wright, J. B. *Trans. R. Soc. N.Z.* **6**, 1 (1968).
26. Wright, J. B. *Trans. R. Soc. N.Z.* **8**, 109 (1970).
27. Hay, R. F., Mutch, A. R. & Watters, W. A. *Bull. N.Z. geol. Surv.* **83** (1970).
28. Grindley, G. W., Adams, C. J. D., Lumb, J. T. & Watters, W. A. *N.Z. J. geol. Geophys.* **20**, 425 (1977).
29. Cullen, D. J. *N.Z. J. geol. Geophys.* **8**, 465 (1965).
30. Weissel, J. K., Hayes, D. E. & Herron, E. M. *Mar. Geol.* **25**, 231 (1977).

## Strontium isotopic resolution of magma dynamics in a layered intrusion

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Kalka is one of about 14 major layered mafic-ultramafic intrusions forming the Giles Complex in central Australia<sup>1</sup>. The stratigraphical section of the Kalka Intrusion passes from a basal pyroxenite zone (450+ m) through norite and olivine gabbro zones (3,500 m) to an uppermost anorthosite zone (800+ m)<sup>2</sup>. Further resolution into 21 cyclic units is derived from repeated mineral crystallization sequences and cyclical variation in plagioclase and olivine compositions. A conventional interpretation would have a basaltic magma progressively fractionating as crystallization proceeded from mafic base to leucocratic top, with periodic resetting to less evolved states by fresh incursions of primary magma or by convectional overturn within a sealed magma chamber. However, the reconnaissance isotopic data reported here indicate development of a more complex open system. High initial <sup>87</sup>Sr/<sup>86</sup>Sr ratios in conjunction with normal <sup>143</sup>Nd/<sup>144</sup>Nd ratios demonstrate massive contamination by country rock within the main part of the intrusion<sup>3</sup>. Furthermore, great variation in Sr initial ratios from 0.7049 to 0.7088 suggests substantial changes in magma composition beyond those induced by fractionation. Similar isotopic heterogeneity in the Newer Gabbros of Scotland<sup>4</sup> and the Bushveld Complex<sup>5</sup> has been attributed to variable contamination or the emplacement of magma batches of differing composition.

The present first detailed examination of the mechanism of isotopic variation in Kalka concentrates on the striking facies relationship between the norite and olivine gabbro zones. The norite zone (NZ) is the major component of the intrusion with a uniform clinopyroxene norite lithology reaching a maximum thickness of 3,500 m. Plagioclase composition shows an overall

upward variation from An<sub>75</sub> to An<sub>60</sub> broadly independent of the substantial separation into lower and upper subzones by the interleaved olivine gabbro zone. The much thinner olivine gabbro zone (OGZ) is totally enclosed within the body of the NZ and has a maximum thickness of 600 m and a strike length of 6.5 km (Fig. 1). It consists of an alternation of orthopyroxene gabbros similar to the rocks of the NZ and lesser olivine gabbro horizons. Plagioclase (An<sub>82-68</sub>) and olivine (Fo<sub>86-69</sub>) compositions oscillate as a function of stratigraphical position although co-crystallizing phases have been separated in the cumulate succession by differential gravity settling<sup>2</sup>. The facies link between the OGZ and NZ is expressed in the former by gradational boundaries definable only on the appearance of olivine, and lateral interfingering of prominent bands into the NZ. The base of the OGZ is displaced to progressively higher stratigraphical levels towards the east and is laterally more extensive at these higher levels (a traditional 'transgressive' stratigraphical relationship in conventional sediments).

Initial <sup>87</sup>Sr/<sup>86</sup>Sr ratios of rocks in a vertical section through the transition from the lower NZ to the OGZ are given in Table 1 and Fig. 2. The boundary is marked by a change from relative uniformity of initial ratio in the lower NZ at ~0.7078 to a cyclical variation in which values fall as low as 0.7068. The upper stratigraphical limit of the cycles lies beyond the sampled interval, but is probably the top contact of the OGZ. Eventually, the overlying section of the upper NZ saw a return to the initial ratio of its lower counterpart in the 0.70784 value of a reconnaissance gabbro-norite sample. Overall, the continuous isotopic fluctuations of the OGZ can be described as a series of downward excursions from the general high value of the NZ. Overall variations in the total NZ itself reveal a small, gradual decrease in initial ratios from 0.7082 to 0.7073 from the base to the top of the zone. The petrological significance of the results is best seen in Fig. 2 through the correspondence of the onset of isotopic variation and the appearance of the olivine that defines the OGZ (olivine is rare in the 1,000 m of the underlying NZ). There is also an antipathetic decline in orthopyroxene, and a partial link between plagioclase and olivine compositional changes and the isotopic curve.

It is axiomatic that variations in Sr isotopic composition are due to physical processes and the only plausible explanation in this case is the mixing of materials with differing <sup>87</sup>Sr/<sup>86</sup>Sr ratios.

While the Nd-Sr study showed that primary basaltic magma and country rock granulite were the ultimate mixing components<sup>3</sup>, the absence of a discrete high-Si phase within the intrusion or other bodies in the Giles Complex points more to an interaction between primary magma (low <sup>87</sup>Sr/<sup>86</sup>Sr ratio) and contaminated magma (high <sup>87</sup>Sr/<sup>86</sup>Sr ratio) as the direct cause of isotopic fluctuations. Because the bulk of rocks within the intrusion show evidence of contamination, it is presumed that contaminated magma dominated the chamber and that primary magma was volumetrically minor and introduced from outside.

Given a low <sup>87</sup>Sr/<sup>86</sup>Sr ratio in primary magma, any phase of decreasing initial ratio leading to an isotopic minimum (A, B, C of Fig. 2) can be attributed to a fresh incursion into the chamber and mixing with the established magma body. Incorporation of primitive magma could also lead to transient undersaturation and short bursts of olivine crystallization at that time (Fig. 2). Increases in initial ratio can be accounted for in two ways. If the

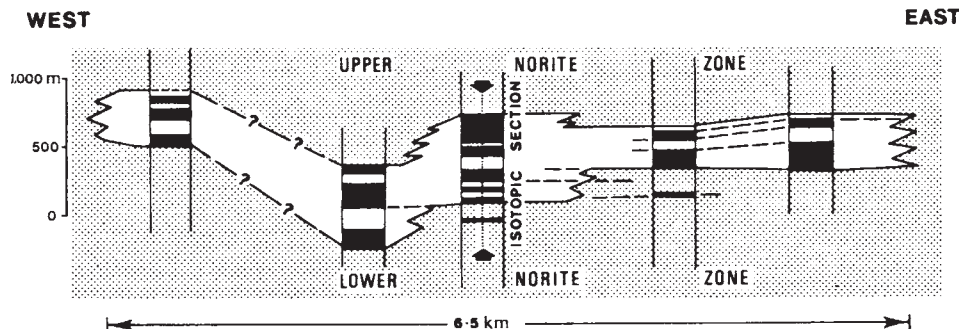
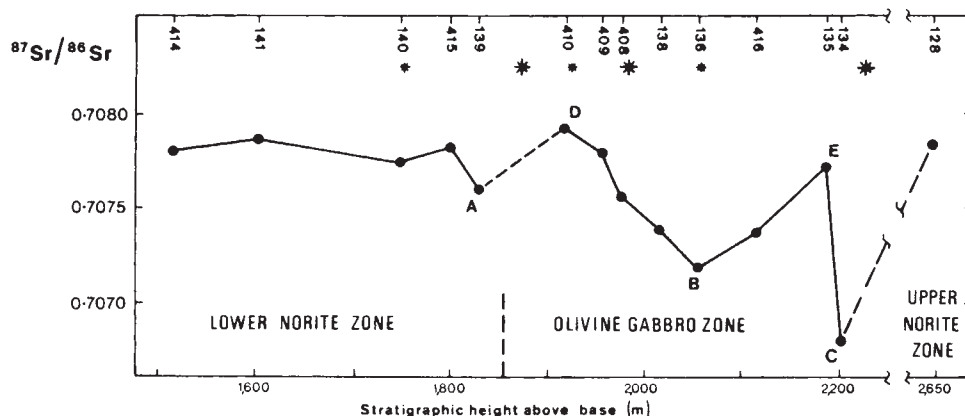


Fig. 1 Schematic facies relationship between norite zone (stippled) and enclosed olivine gabbro zone (OGZ) in the Kalka Intrusion. Olivine-bearing units shown in black. Note increased lateral extent and eastward migration of OGZ with time.



**Fig. 2** Isotopic variation in the transition from the lower norite zone to the olivine gabbro zone, Kalka Intrusion. Sample numbers are shown along the top axis (major olivine component indicated by large asterisk, minor olivine component by small asterisk). Location of the section is shown in Fig. 1.

magma body were continuously mixed and isotopically homogeneous at any one time, the only explanation would involve addition from outside of material with a higher  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio, either as direct contamination by wall rock or an influx of more contaminated magma. Accordingly, fluctuations in isotopic composition would reflect the interplay between the low and high  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio sources; but while plausible, such a mechanism would probably produce random variations in contrast to the more regular observed pattern. Rather, the periodic return to a relatively constant isotopic ratio suggests that the new magma pulses were interacting with an almost infinite reservoir of contaminated magma; that they initially affected isolated aliquots of this reservoir, but were eventually absorbed by the reservoir. Increases in initial ratio, such as to D and E of Fig. 2, would then be due to a return to the background value of the intrusion.

The preferred model begins at the end of lower NZ time with the Kalka magma chamber filled by a well mixed contaminated basaltic liquid of relatively low density ( $<2.66$ )<sup>6</sup> as a result of considerable crystallization of the pyroxene that accumulated in the pyroxenite zone; it is precipitating the two pyroxenes and plagioclase of the NZ with the high  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio of 0.7078. A small pulse of primary magma enters the chamber and floods the floor due to its higher density. In moving across the floor the influx partly mixes with the adjacent resident magma forming a hybrid, which, still with a higher density than the main body of magma, forms an isolated layer that dominates the basal crystallization zone (evidence for basal crystallization in Kalka has been presented elsewhere<sup>2</sup>). The precipitated phases now reflect the composition of the hybrid:  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios decrease and a short burst of olivine deposition as an olivine gabbro layer marks the beginning of crystal fractionation. The details of the petrological changes induced are determined by the nature of

the magma influxes. For example, sequential small pulses over a period of time will produce the gradual decline to minimum B (Fig. 2) whereas a single major pulse produces the abrupt drop to C.

The facies relationship between the OGZ and NZ is due to the restricted ponding of the magma pulses at low points on the floor of the intrusion: at any stage only part of the accumulative interface is disturbed and changes in the position of the low points with time can account for the eastern lateral migration of the main OGZ up-section (Fig. 1). The correspondence of the maximum lateral extent of the OGZ, the most magnesian olivine and the most calcic plagioclase, and the minimum initial  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios in the upper parts of the OGZ are consistent with the greatest relative volumes of fresh magma injections being added to the system at that time. Ultimately, convective mixing within the chamber as a whole disperses the residual hybrid, with crystallization reverting to the initial NZ state to await further magma pulses. At the same time, incorporation of the hybrid slightly lowers the background  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio of the main body of magma.

Independently, Huppert and Sparks<sup>7,8</sup> have proposed a similar model on fluid dynamical grounds thereby providing the physical basis of the processes described above. In particular, they demonstrate the formation of a stable basal layer by the incoming batch of magma and the eventual convective dispersal of the layer on cooling when density equilibration occurs. The link between petrological, fluid dynamic and isotopic approaches augurs well for the future elaboration of the evolution of layered intrusions and can give a more specific explanation of the origin of some types of macroscopic layering and cyclic units.

We conclude that the lithological transition from a monotonous gabbro-norite sequence to a zone of vertically and

**Table 1** Rb-Sr analytical data for Kalka igneous rocks

| Sample | Lithology             | Rb<br>(p.p.m.) | Sr<br>(p.p.m.) | $^{87}\text{Rb}/^{86}\text{Sr}$ | $^{87}\text{Sr}/^{86}\text{Sr}$<br>present | $^{87}\text{Sr}/^{86}\text{Sr}$<br>1,150 Myr |
|--------|-----------------------|----------------|----------------|---------------------------------|--------------------------------------------|----------------------------------------------|
| 414    | Melagabbro-norite     | 1.17           | 73.1           | 0.0464                          | $0.70855 \pm 4$                            | 0.70779                                      |
| 141    | Leuconorite           | 1.24           | 226.8          | 0.0158                          | $0.70811 \pm 6$                            | 0.70785                                      |
| 140    | Olivine gabbro-norite | 1.10           | 133.7          | 0.0238                          | $0.70812 \pm 5$                            | 0.70773                                      |
| 415    | Melagabbro            | 0.43           | 108.9          | 0.0115                          | $0.70801 \pm 6$                            | 0.70782                                      |
| 139    | Gabbro                | 0.39           | 106.5          | 0.0107                          | $0.70777 \pm 4$                            | 0.70759                                      |
| 140    | Olivine gabbro        | 0.59           | 102.7          | 0.0165                          | $0.70818 \pm 3$                            | 0.70791                                      |
| 409    | Gabbro-norite         | 0.87           | 116.8          | 0.0216                          | $0.70814 \pm 4$                            | 0.70778                                      |
| 408    | Olivine melagabbro    | 0.73           | 63.8           | 0.0332                          | $0.70810 \pm 3$                            | 0.70755                                      |
| 138    | Gabbro-norite         | 0.88           | 141.1          | 0.0181                          | $0.70768 \pm 4$                            | 0.70738                                      |
| 136    | Olivine gabbro        | 0.50           | 98.1           | 0.0146                          | $0.70742 \pm 4$                            | 0.70718                                      |
| 416    | Melagabbro            | 0.61           | 82.2           | 0.0216                          | $0.70773 \pm 4$                            | 0.70737                                      |
| 135    | Gabbro-norite         | 0.58           | 114.2          | 0.0146                          | $0.70795 \pm 8$                            | 0.70771                                      |
| 134    | Anorthosite           | 0.31           | 254.7          | 0.0035                          | $0.70683 \pm 6$                            | 0.70679                                      |
| 128    | Gabbro-norite         | 1.27           | 165.5          | 0.0222                          | $0.70821 \pm 6$                            | 0.70784                                      |

Sr isotopic ratios were measured at La Trobe University using a 22-cm radius solid-source mass spectrometer of modified Clement design. Uncertainties quoted in present-day  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios are  $\pm 2\sigma$  mean. Measurements are relative to a value of  $0.71022 \pm 4$  for the SRM 987 Sr standard. Initial ratios are calculated for an age of 1,150 Myr using  $\lambda^{87}\text{Rb} = 1.42 \times 10^{-11} \text{ yr}^{-1}$ . The age is an arbitrary mean for the 1,200–1,100 Myr time interval for emplacement of the Kalka Intrusion.



laterally restricted olivine gabbro horizons in Kalka correlates with the onset of cyclic fluctuations in initial  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios. Isotopic variability is attributed to dynamic mixing between the main body of contaminated magma in the chamber and incoming pulses of primary basaltic liquid. New magma batches ponded on the floor of the chamber temporarily dominating part of the basal crystallization zone; the result, transient olivine precipitation in laterally limited olivine gabbro layers having low Sr initial ratios. Ultimately, convective dispersal of the pools returned crystallization to its initial state with an accompanying rise in initial  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios to original or slightly reduced values.

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1. Nesbitt, R. W., Goode, A. D. T., Moore, A. C. & Hopwood, T. P. *Spec. Publ. geol. Soc. S. Afr.* **1**, 547–564 (1970).
2. Goode, A. D. T. *J. Petrol.* **17**, 379–397 (1976).
3. Gray, C. M., Cliff, R. A. & Goode, A. D. T. *Earth planet. Sci. Lett.* (in the press).
4. Pankhurst, R. J. *J. Petrol.* **10**, 115–143 (1969).
5. Hamilton, J. J. *Petrol.* **18**, 24–52 (1977).
6. Goode, A. D. T. *Earth planet. Sci. Lett.* **34**, 375–380 (1977).
7. Huppert, H. E. & Sparks, R. S. J. *Nature* **286**, 46–48 (1980).
8. Huppert, H. E. & Sparks, R. S. J. *Contr. Mineral. Petrol.* **75**, 279–289 (1980).

## Shallowing-upward cycles in the Middle Proterozoic Altyn Formation

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**Phanerozoic carbonate rocks commonly contain cycles, a few metres thick, which were deposited during progressive shallowing of their oceanic environment<sup>1</sup>. Each cycle represents a deepening of water in a nearshore area, followed by the accumulation of sediments up to sea level. The causes of the water deepening which begins each cycle are rarely known. Possibilities include tectonic activity that affected the depositional basin and changes in ocean volume due to the expansion and contraction of ice sheets. I report here that similar cycles occur in the Middle Proterozoic Altyn Formation, part of the Belt Supergroup<sup>2</sup>. The cycles are probably related to vertical tectonic activity which influenced sedimentation in the Belt basin. The presence of shallowing-upward cycles in the Altyn Formation shows that such cycles are not confined to the Phanerozoic. Other Precambrian sequences should be examined to see if they too contain cycles. Where such cycles are found they may give important information about Precambrian tectonism and glaciation.**

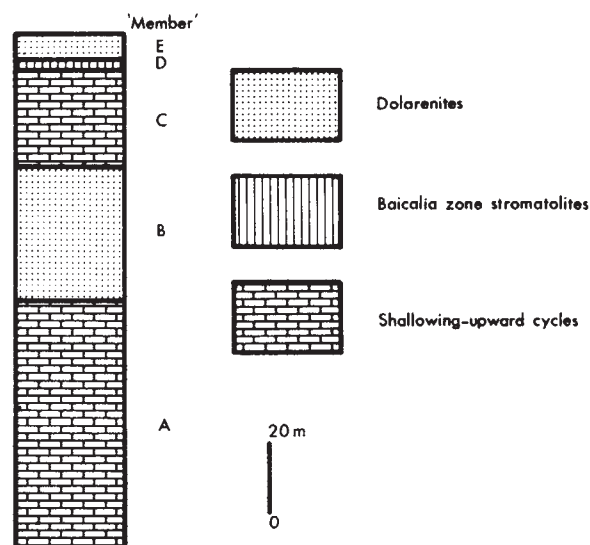
The Altyn Formation is exposed in the eastern part of Glacier National Park, Montana. The age of the formation may be estimated from geochronological data available for equivalent stratigraphic units. The Aldridge Formation of British Columbia has granodiorite intrusives which are at least 1,335 Myr old<sup>3</sup> and a sill in the lower part of the Prichard Formation in Idaho is 1,430 Myr old (R. E. Zartman, unpublished data). A best estimate of the age of the Altyn Formation is  $1,450 \pm 50$  Myr (J. Harrison, personal communication).

I have divided the Altyn Formation into the informal members shown in Fig. 1. Seventeen cycles have been identified in member A with an average thickness of 4.2 m and a range of 1.6–8.0 m. Member C has 12 cycles ranging from 1.6 to 4.6 m thick with an average of 2.5 m. Members B and E are quite homogeneous and devoid of stromatolites or other palaeo-environmentally diagnostic sedimentary structures, and so far no cycles have been recognized in them.

An idealized and simplified version of the Altyn Formation cycles is shown in Fig. 2. The cycles are bounded by erosion surfaces. The lowest part of each cycle usually has

conglomerates and breccias composed of stromatolite fragments and pebbles of quartz and microcline. Dolarenites composed of peloids and intraclasts with variable amounts of quartz and microcline overlie the coarse basal layer. These dolarenites occasionally contain tabular cross-bedding. In the upper part very similar dolarenites alternate with stratiform stromatolites. The dolarenites commonly contain lenticular bedding and bimodal cross-bedding produced by the alternating flow of tidal currents. The stromatolites are very similar to those formed by smooth algal mats in modern lower intertidal zones of Shark Bay<sup>4,5</sup> and the Persian Gulf<sup>6</sup>. Smooth mat development is inhibited in Shark Bay and the Persian Gulf in areas where sand substrates are kept moving by strong tidal currents and waves<sup>5,6</sup>. Similarly the stratiform stromatolites of the Altyn Formation formed during relatively low energy conditions in the intertidal zone. This development of stromatolites was interrupted periodically by higher energy events, such as storms, which brought in sands from the adjacent subtidal zone. There is usually an upward increase in the ratio of stromatolites to dolarenites in each cycle, showing the decreasing influence of sand input from subtidal areas. Many stromatolites in the upper part of cycles have desiccation cracks and their upper surfaces are broken and disrupted to form intraformational breccias. These features are the result of increasing subaerial emergence and consequent drying of the sediments during the development of the upper part of the cycles. Each cycle represents deposition in progressively shallower water as sediments built up to sea level following an increase in water depth. Subtidal sands are overlain by lower intertidal sands and stromatolites which in turn pass upward into upper intertidal and supratidal stromatolites and intraformational breccias and conglomerates. This sequence is typical of cycles found in many Phanerozoic carbonate rocks<sup>1</sup>. There are few reported examples of shallowing-upward cycles from rocks of Precambrian age. One possibility is the Strelley Pool chert which occurs in the 3,400-Myr old Warrawoona Group of Western Australia. This is an evaporite-carbonate sequence containing stromatolites. The Strelley Pool chert was deposited in shallow subtidal to intertidal environments<sup>7</sup>, and may represent an overall shallowing-upward sequence.

There are two important modifications of the idealized shallowing-upward cycle described above. Member A frequently contains discontinuous bioherms of columnar stromatolites near the base of the stromatolite-rich part of cycles. These commonly occur with conglomerates and breccias and overlie erosion surfaces. The columnar stromatolites were formed in localized higher energy environments in the lower intertidal zone, especially in tidal channels<sup>8</sup>. Many member C cycles contain arkoses



**Fig. 1** Altyn Formation lithostratigraphy at the type section in the Appekunny Creek area, Glacier National Park, Montana.

and shales, mainly in their upper parts. These terrigenous sediments were deposited in the upper intertidal and supratidal zones during flash flooding from the adjacent land area.

Further support for shallowing-upward depositional conditions in Altyn Formation cycles is provided by the former presence of evaporite minerals and the dolomitization of carbonate minerals. The evidence for evaporites includes silica pseudomorphs after gypsum crystals and anhydrite nodules, often with micrometre-sized gypsum and anhydrite preserved in the silica. The pseudomorphs contain length-slow chalcedony and flamboyant quartz, both of which replace evaporite minerals<sup>9,10</sup>.

Cellular vugs filled by a centripetal sequence of silica, dolomite and calcite also occur. Some of these have a 'chicken-wire' fabric which is characteristic of many modern and ancient anhydrite nodules<sup>10</sup>. The formation of displacive gypsum crystals and anhydrite nodules is a diagenetic process which takes place in sediments as a result of intense evaporation of interstitial water in arid climates<sup>1,11</sup>. Gypsum crystals grow within sediments in the upper intertidal zone and anhydrite forms in the supratidal zone<sup>11</sup>. Sediments deposited in shallow subtidal or lower intertidal zones may be exposed later to upper intertidal or supratidal zone conditions as a result of progradation. Evaporites may form in these sediments because of this change of environment. The precipitation of calcium sulphate evaporite minerals in these sediments produces magnesium-rich interstitial waters which may lead to dolomitization of carbonate minerals<sup>1,11</sup>. In the Altyn Formation some columnar stromatolites and lower intertidal sediments contain replaced evaporites, showing that they were subsequently exposed to upper intertidal and supratidal conditions. All of the original carbonate minerals in each of the cycles are dolomitized as a result of these changes of environment. The dolomitization and formation of evaporites could have occurred only in shallowing-upward cycles following sediment aggradation and the resultant seaward shift of the tidal zones.

Detailed studies show that many Phanerozoic carbonate rocks are cyclic and that each cycle represents aggradation during progradation, following a rapid transgression during which little sediment was deposited<sup>1,12,13</sup>. The accumulation of a sequence of shallowing-upward cycles requires repeated transgressions, the cause of which is not well understood. Some possibilities include differential subsidence due to tectonic activity, eustatic sea level changes resulting from glaciation or changes in ocean volume caused by global tectonics<sup>14</sup>. The period 2,300–950 Myr ago was characterized by warm climates, with no evidence of glacial periods<sup>15</sup>. Thus glacial eustasy seems not to be the cause of cyclic sedimentation in the Altyn Formation. Opinions differ regarding the tectonic setting of the Belt depositional basin. One

view is that the basin was located at a rifted continental margin of the passive or Atlantic type<sup>16–18</sup>, with vertical tectonic activity during deposition<sup>18</sup>. An opposing view suggests that the Belt basin was one of several, possibly fault-bounded, basins located within a continental craton<sup>19,20</sup>.

Major contemporaneous faulting at the southern margin of the Belt basin strongly influenced sedimentation of the coarse-grained LaHood Formation<sup>21</sup>. This formation is equivalent in age to the Altyn Formation. Thus cyclic sedimentation in the Altyn Formation was probably related to intermittent subsidence caused by vertical tectonic activity, with sediment accumulation occurring in quieter periods between the tectonic events. Sedimentation elsewhere in the Belt basin was probably affected by tectonic activity. Detailed studies of other stratigraphic units of the Belt Supergroup may reveal cycles or manifestations of this tectonism. The presence of small-scale cycles in the Altyn Formation suggests that Precambrian sequences in other localities should be examined closely to see if they also contain cycles.

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1. James, N. P. in *Facies Models* Vol. 1 (ed. Walker, R. G.) 109–119 (Geoscience Canada, Reprint Series, 1979).
2. Harrison, J. E. *Bull. geol. Soc. Am.*, **83**, 1215–1240 (1972).
3. Obradovich, J. D. & Peterman, Z. E. *Belt Symp.* 1, 8–9 (Idaho Bureau of Mines, Moscow, Idaho, 1973).
4. Davies, G. R., *Am. Ass. petrol. Geol. Mem.* **13**, 169–205 (1970).
5. Logan, B. W., Hoffman, P. & Gebelein, C. D. *Am. Ass. petrol. Geol. Mem.* **22**, 140–194 (1974).
6. Kinsman, D. J. J. & Park, R. K. *Stromatolites* (ed. Walter, M. R.) 421–433 (Elsevier, Amsterdam, 1976).
7. Lowe, D. R. *Nature* **284**, 441–443 (1980).
8. White, B. *Geol. Soc. Am., Abstr. Progr.* **5**, 452–453 (1973).
9. Folk, R. L. & Pittman, J. S. *J. Sediment. Petrol.* **41**, 1045–1058 (1971).
10. Tucker, M. E. *Sediment. Geol.* **16**, 193–204 (1976).
11. Kendall, A. C. in *Facies Models* Vol. 1 (ed. Walker, R. G.) 145–157 (Geoscience Canada Reprint Series, 1979).
12. Wilson, J. L. *Carbonate Facies in Geologic History*, 281–318 (Springer, New York, 1975).
13. Anderson, E. J. & Goodwin, P. W. *Geol. Soc. Am., Abstr. Progr.* **12**, 21–22 (1980).
14. Anderson, E. J. and Goodwin, P. W. *Soc. Econ. Paleontol. Miner., Eastern Sect., Field Conf. Guidebook*, 16–17 (1980).
15. Frakes, L. A. *Climates through Geologic Time*, 49–55 (Elsevier, Amsterdam, 1979).
16. Badham, J. P. N. *Geology* **6**, 621–625 (1978).
17. Sears, J. W. & Price, R. A. *Geology* **6**, 267–270 (1978).
18. Harrison, J. E. & Reynolds, M. W. *Geol. Soc. Am. Abstr. Progr.* **8**, 905 (1976).
19. Stewart, J. H. *Geology* **4**, 11–15 (1976).
20. Young, G. M., Jefferson, C. W., Delaney, G. D. & Yeo, G. M. *Geology* **7**, 125–128 (1979).
21. McMannis, W. J. *Bull. geol. Soc. Am.*, **74**, 407–436 (1963).

## Superoxide dismutase is a prophylactic against alloxan diabetes

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A single injection of alloxan<sup>1</sup>, or alloxan-like derivatives of uric acid<sup>2,3</sup>, kills the insulin-producing islet  $\beta$ -cells and causes diabetes mellitus in animals. This effect is probably mediated by a sequence of redox reactions involving the production of superoxide anion radicals in or near the  $\beta$ -cells<sup>4–8</sup>. Superoxide anions may also arise in inflammation<sup>9–15</sup>, and islet inflammation often accompanies the outbreak of human diabetes<sup>16</sup>, perhaps following the combined onslaught of viruses and chemical agents on the  $\beta$ -cells<sup>17</sup>. We now report that injections of superoxide dismutase, an enzyme removing superoxide anion radicals, act prophylactically against alloxan-induced diabetes.

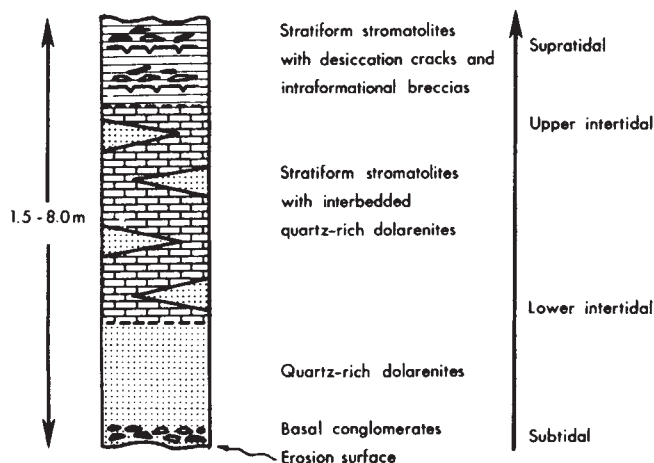
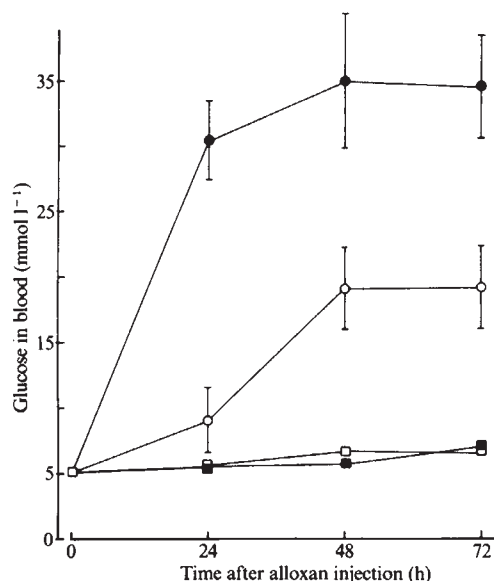


Fig. 2 Simplified shallowing-upward cycle representative of those found in the Altyn Formation.





**Fig. 1** Blood glucose concentrations in uninjected mice (0 h) and in mice intravenously injected with 25 (■), 50 (○) or 75 (▲) mg alloxan per kg body weight. Alloxan monohydrate (U.S. Biochemical Corporation) was dissolved in a salt-balanced Krebs–Ringer buffer that had been adjusted to pH 4.0 by the addition of HCl. Female 3-month-old non-inbred mice (21–26 g) were injected into a tail vein with alloxan solution at a dosage of 100 µl per 20 g mouse; control mice were similarly injected with physiological saline (□). Blood samples (25 µl each) were taken from the cut tail tips 24, 48 and 72 h after injection, precipitated by ZnSO<sub>4</sub> and NaOH and assayed by an automatic glucose oxidase/peroxidase technique (Glox reagents; Kabi). Each point is the mean for five different mice; vertical bars show s.e.m. when larger than the symbol indicating the mean value.

When mice were injected into a tail vein with alloxan (50 or 75 mg per kg body weight), they became markedly hyperglycaemic within 48 h (Fig. 1). The diabetogenic effect was clearly dose-dependent, as alloxan at 25 mg per kg had no effect and 50 mg per kg produced a milder hyperglycaemia than did 75 mg per kg. With increasing concentrations, alloxan attacks cells other than the islet  $\beta$ -cells, for example, kidney and liver cells<sup>1</sup>. The unequivocal, though not maximal, diabetogenic action of 50 mg per kg would provide optimum conditions for detecting any protecting or potentiating effect of the substances to be tested, and so this intermediate alloxan dose was selected for the subsequent experiments.

As shown in Table 1, in each of two separate series of experiments the diabetogenic action of alloxan was largely prevented if the mice had been injected with superoxide dismutase 12 h earlier. The enzyme preparation injected (provided by K.-E. Arfors, Pharmacia) was a purified yeast CuZn-superoxide dismutase covalently linked to polyethylene glycol chains to yield a complex of molecular weight 100,000, so as to slow down its elimination from the circulation<sup>18</sup>. Whereas the circulating half life of uncoupled superoxide dismutase in the rat is only 6 min (ref. 14), the enzyme–polyethylene glycol complex had an initial half life of about 2 h (Fig. 2). The elimination was more complicated than a first-order process, and after 12 h as much as 6% of the initial activity remained in the circulation. This remaining activity was about 10 times the basal activity of superoxide dismutase measured in the plasma of four untreated control mice ( $505 \pm 19$  U ml<sup>-1</sup>; mean  $\pm$  s.e.m.).

The antidiabetogenic action of superoxide dismutase was specific, as control experiments revealed no effect of inactivated superoxide dismutase–polyethylene glycol or of polyethylene glycol alone (Table 1). Furthermore, the enzymatically active superoxide dismutase–polyethylene glycol had no effect on blood glucose levels in healthy mice not treated with alloxan (Table 1).

According to the free-radical hypothesis of alloxan diabetogenicity, reduction of alloxan to dialuric acid in the islets is followed by autooxidation back to alloxan accompanied by the formation of superoxide anion radicals and hydrogen peroxide<sup>4–8</sup>. Highly reactive hydroxyl radicals are then formed by a metal-catalysed 'Haber–Weiss reaction' that can be seen as the summation of the reduction of ferric ion by superoxide ( $\text{Fe}^{3+} + \text{O}_2^{\cdot-} \rightarrow \text{Fe}^{2+} + \text{O}_2$ ) and the Fenton reaction ( $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH} \cdot + \text{OH}^- + \text{Fe}^{3+}$ ). The extreme and indiscriminatory reactivity of hydroxyl radicals can explain why alloxan rapidly affects a wide range of phenomena in the  $\beta$ -cells, including insulin synthesis<sup>19</sup>, insulin release<sup>8,20</sup>, cation pumping<sup>6,21</sup>, glucose metabolism and oxygen consumption<sup>22</sup>, Trypan blue exclusion<sup>6,23</sup> and mitochondrial morphology<sup>24</sup>. In support of this hypothesis alloxan toxicity is counteracted *in vivo* by scavengers of hydroxyl radicals<sup>5</sup> and *in vitro* by such scavengers<sup>6,25</sup> as well as by superoxide dismutase, catalase and a chelator of iron ions<sup>6–8</sup>. The present results represent further, and crucial, support for the hypothesis, as they confirm the

**Table 1** Prophylaxis by superoxide dismutase against alloxan diabetes in mice

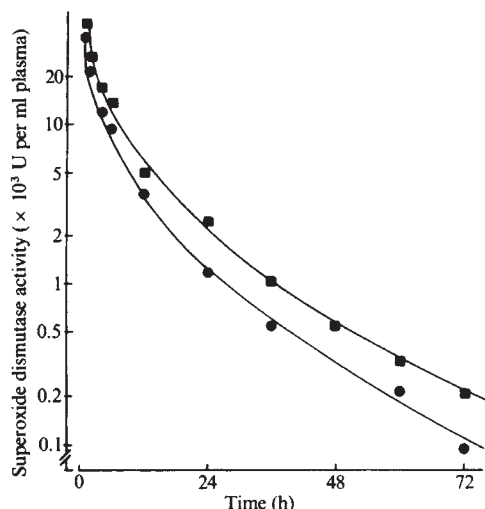
| Drug injected before<br>alloxan or NaCl | Time between<br>injections<br>of SOD/PEG<br>(or PEG)<br>and alloxan | Blood glucose (mmol l <sup>-1</sup> )<br>Alloxan | NaCl          |
|-----------------------------------------|---------------------------------------------------------------------|--------------------------------------------------|---------------|
| <i>First experimental series</i>        |                                                                     |                                                  |               |
| None                                    | —                                                                   | 20.4 ± 2.4 (5)                                   | 4.8 ± 0.1 (9) |
| SOD-PEG, 200 mg per kg                  | 12 h                                                                | 9.2 ± 1.4* (6)                                   | 5.0 ± 0.2 (4) |
| Inactivated SOD-PEG,<br>200 mg per kg   | 12 h                                                                | 21.1 ± 2.5 (5)                                   | 5.2 ± 0.2 (5) |
| <i>Second experimental series</i>       |                                                                     |                                                  |               |
| None                                    | —                                                                   | 21.4 ± 2.4 (6)                                   | 4.8 ± 0.3 (4) |
| SOD-PEG, 250 mg per kg                  | 12 h                                                                | 8.2 ± 2.1* (6)                                   | 5.7 ± 0.1 (6) |
| Inactivated SOD-PEG,<br>250 mg per kg   | 12 h                                                                | 20.4 ± 1.9 (6)                                   | 5.4 ± 0.2 (6) |
| PEG, 250 mg per kg                      | 12 h                                                                | 19.0 ± 1.1 (5)                                   | 5.6 ± 0.3 (5) |
| PEG, 250 mg per kg                      | 2 h                                                                 | 17.9 ± 1.2 (5)                                   | 5.1 ± 0.3 (5) |

Mice were injected with alloxan (50 mg per kg) or NaCl, and blood glucose was assayed after 72 h as in Fig. 1. Before injection with alloxan or NaCl, groups of mice were injected into a tail vein with yeast CuZn-superoxide dismutase coupled to polyethylene glycol (SOD-PEG), with SOD-PEG that had lost 99.2% of its enzyme activity during prior exposure to H<sub>2</sub>O<sub>2</sub> (ref. 6), or with uncoupled polyethylene glycol alone (PEG). The time elapsing between the injection of SOD-PEG or PEG on the one hand and alloxan or NaCl on the other was 12 or 2 h as indicated. All mice were fasted, with free access to water for 12 h before the alloxan or NaCl injection and for 3 h before the blood sampling. Results are given as mean values  $\pm$  s.e.m. for the numbers of animals stated in parentheses. In the two separate series, different batches of SOD-PEG were used.

\* The prophylactic effect of SOD-PEG against alloxan diabetes was significant, with  $P < 0.01$  in either series of experiments (Student's *t*-test, two-tailed). Among the 11 mice injected with alloxan alone, the lowest and highest blood glucose values were 11.2 and 28.1 mmol l<sup>-1</sup>. The corresponding range for the 13 mice injected with NaCl alone was 3.9–5.5 mmol l<sup>-1</sup>. Among the 12 mice injected with alloxan after 200 or 250 mg per kg SOD-PEG, as many as 6 had blood glucose values in the range of 4.7–5.9; the remaining 6 values ranged over 8.5–17.0.

postulated role of superoxide anion radicals in the diabetogenic action of alloxan *in vivo*. Even more important, apparently for the first time it has been possible to protect against alloxan diabetes by administering a drug several hours beforehand. This result may be of more than theoretical interest, as superoxide dismutase has already been established as a safe drug of potential value for the treatment of other diseases in humans<sup>26</sup>.

It has been suggested that the superoxide anion radical is relatively harmless *in vivo*<sup>27,28</sup>, based on the relatively low reactivity of the radical with several classes of compound *in vitro*, and the assumption that other reductants might substitute for it in reducing the ferric ion in the Haber–Weiss reaction. Against this background our results seem important in demonstrating the high toxic potential of the superoxide radical *in vivo*, as well as in underlining the importance of an adequate protection against it. The results do not necessarily mean that  $\beta$ -cells are



**Fig. 2** Superoxide dismutase activity in the blood plasma of two mice at various times after an intravenous injection of yeast CuZn-superoxide dismutase coupled to polyethylene glycol; the drug was injected into a tail vein at a dosage of 200 mg per body weight. Samples (25  $\mu$ l each) of whole blood were drawn from the tails and mixed with 500  $\mu$ l of a solution containing 150 mmol l<sup>-1</sup> NaCl and 3 mmol l<sup>-1</sup> EDTA. After centrifugation, the supernatant was assayed for superoxide dismutase by the direct spectrophotometric method using KO<sub>2</sub> (ref. 29). One unit of enzyme activity corresponds to 4.2 ng of bovine CuZn-superoxide dismutase. Plasma levels were calculated by assuming an erythrocyte blood volume fraction of 40%. To show the elevation of enzyme activity produced by injecting SOD-PEG, the values in the figure have been corrected for the mean endogenous superoxide dismutase activity in mouse plasma (505 U ml<sup>-1</sup>).

especially sensitive to alloxan because they are extraordinarily sensitive to the harmful effects of superoxide radicals. It seems equally possible that the physiological specialization of the  $\beta$ -cells renders them unusually effective in reducing alloxan or in catalysing the iron-dependent formation of OH $\cdot$  from H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>.

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1. Rerup, C. C. *Pharmac. Rev.* **22**, 485-518 (1970).
2. Poje, M. & Rđić, B. *Tetrahedron Lett.* **49**, 4781-4782 (1979).
3. Poje, M. & Rđić, B. *Experientia* **36**, 78-79 (1980).
4. Deamer, D. W., Heikkilä, R. E., Panganamala, R. V., Cohen, G. & Cornwell, D. G. *Physiol. chem. Phys.* **3**, 426-430 (1971).
5. Heikkilä, R. E., Winston, B., Cohen, G. & Barden, H. *Biochem. Pharmac.* **25**, 1085-1092 (1976).
6. Grankvist, K., Marklund, S., Sehlin, J. & Täljedal, I.-B. *Biochem. J.* **182**, 17-25 (1979).
7. Grankvist, K., Marklund, S. & Täljedal, I.-B. *FEBS Lett.* **105**, 15-18 (1979).
8. Fischer, L. J. & Hamburger, S. A. *Diabetes* **29**, 213-216 (1980).
9. Babior, B. M., Kipnes, R. S. & Curnutte, J. T. *J. clin. Invest.* **52**, 741-744 (1973).
10. Johnston, R. B., Lehmyer, J. E. & Guthrie, L. A. *J. exp. Med.* **143**, 1551-1556 (1976).
11. DeChatelet, L. R. *et al. Blood* **50**, 525-535 (1977).
12. Johnston, R. B., Godzik, C. A. & Cohn, Z. A. *J. exp. Med.* **148**, 115-127 (1978).
13. Sacks, T., Moldow, C. F., Craddock, P. R., Bowers, T. K. & Jacob, H. S. *J. clin. Invest.* **62**, 1161-1167 (1978).
14. McCord, J. M. & Wong, K. *Ciba Fdn Symp.* **65**, 343-351 (1979).
15. Petrone, W. F., English, D. K., Wong, K. & McCord, J. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1159-1163 (1980).
16. Gepts, W. *Acta endocr., Copenh.* **83**, Suppl. 205, 95-104 (1976).
17. Toniolo, A., Onodera, T., Yoon, J.-W. & Notkins, A. L. *Nature* **288**, 383-385 (1980).
18. Payatak, P. S., Abuchowsky, A. & Davis, F. F. *Res. Commun. chem. Path. Pharmac.* **29**, 113-127 (1980).
19. Gunnarsson, R. *Molec. Pharmac.* **11**, 759-765 (1975).
20. Lacy, P. E., McDaniel, M. L., Fink, C. J. & Roth, C. *Diabetologia* **11**, 501-507 (1975).
21. Idahl, L.-A., Lernmark, Å., Sehlin, J. & Täljedal, I.-B. *Biochem. J.* **162**, 9-18 (1977).
22. Borg, H., Eide, S. J., Andersson, A. & Hellerström, C. *Biochem. J.* **182**, 797-802 (1979).
23. Grankvist, K., Lernmark, Å. & Täljedal, I.-B. *J. endocr. Invest.* **2**, 139-145 (1979).
24. Lorentzon, R. & Boquist, L. *Virchows Arch. path. Anat. Physiol.* **31**, 227-233 (1979).
25. Fischer, L. J. & Hamburger, S. A. *Life Sci.* **26**, 1405-1409 (1980).
26. Menander-Huber, K. B. in *Biological and Clinical Aspects of Superoxide and Superoxide Dismutase* (eds Bannister, W. H. & Bannister, J. V.) 408-423 (Elsevier, New York, 1980).
27. Fee, J. A. in *Biological and Clinical Aspects of Superoxide and Superoxide Dismutase* (eds Bannister, W. H. & Bannister, J. V.) 41-48 (Elsevier, New York, 1980).
28. Winterbourn, C. C. *Biochem. J.* **182**, 625-628 (1979).
29. Marklund, S. *J. biol. Chem.* **251**, 7504-7507 (1976).

## Nepotism among rhesus monkey brothers

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**Either during or shortly after sexual maturation, male rhesus monkeys leave the social groups in which they were born and join other non-natal groups (refs 1, 2 and unpublished data). Here we report that males frequently transfer into the same social groups as their older maternally related brothers. Such brothers spent more time close to each other than to other males, formed alliances with brothers more frequently than with non-brothers and disrupted each other's interactions with oestrous females less frequently than expected by chance. They also spent more time in non-natal groups than did males who did not have a brother in the group. Length of time in a group is positively associated with male dominance rank<sup>1</sup> and probably with reproductive success. The data are consistent with proposals that brothers who behave nepotistically and noncompetitively have high inclusive fitness.**

Data are presented from long-term census records and 339 h of focal-animal<sup>3</sup> observations (February 1978-February 1979) of 18 male rhesus monkeys who comprised seven sets of siblings: a quintet, a triad and five pairs. The subjects lived in four social groups containing ~200, 175, 160 and 110 animals (groups A, I, C and E, respectively). The four study groups and four other social groups were free-ranging on La Cueva, a provisioned 100-acre island off the south-west coast of Puerto Rico<sup>4</sup>. Three of the four study groups were introduced to the island in 1962, the fourth in 1969 and four smaller groups in 1975. One other group was introduced in 1962, but decreased in size and disintegrated by 1975. Each monkey had an identification tattoo of letters and numbers and the maternal relations were known for all animals in the four study groups<sup>4</sup>.

During 20-min observation periods, the frequency and duration of approaches within 2 m (excluding agonistic encounters) between the focal male and all other individuals in his group were recorded, as were all his involvements in agonistic encounters and his interactions with oestrous females. All but three of the subjects were observed for 51 periods. Two subjects died (G6 and N6, 21 and 33 observations, respectively) and one (CT5, 30 observations) was observed from mid-study. By comparing each subject's interactions with brothers and non-brothers during only his set of observations, no bias is introduced by unequal samples of observations. For all subjects, equal numbers of observations were made during each of two time periods: 06.30-09.00 and 09.00-11.30 h.

Analysis of group transfer among young males suggested that they shifted into groups containing older brothers. Although males transfer preferentially into the non-natal group with the highest ratio of adult females to adult males<sup>1</sup>, less than half of the males that have ever joined a brother's group on La Cueva ( $n = 17$ ) joined the non-natal group with the highest ratio. Each social group on La Cueva often came into contact with all other groups<sup>5</sup> and there was no evidence that geographical location of groups influenced group transfer. In addition, no male who was transferring from his natal group had a brother in more than one non-natal group.

If we assume the distribution of males in non-natal groups to be random, the probability of a male's joining his brother(s) in



one of three study groups was 0.33. This expected probability was an overestimate for some males, because after 1975 there were four small groups that they could have joined (which did not contain older brothers of any transferring males). By assuming a larger probability, however, it is more difficult to reject the null hypothesis that group transfer is independent of kinship. Since 1962, there have been 31 males who had a brother in a non-natal group when they changed groups. Seventeen joined their brothers' groups while 14 did not ( $\chi^2 = 6.5$ , d.f. = 1,  $P < 0.05$ ). At the end of the study, 9 of 14 living males who had left their natal group and had an older brother in a non-natal group were in their older brothers' groups ( $\chi^2 = 6.2$ , d.f. = 1,  $P < 0.05$ ). Males from the same natal groups were not significantly clumped ( $\chi^2 = 13.69$ , d.f. = 11,  $P > 0.10$ ).

Fourteen of the 18 subjects approached their brother(s) more frequently than they approached any other males ( $P < 0.05$ , sign test), and brothers spent more time within 2 m of each other ( $\bar{x} = 24.5$  min) than of any other males ( $\bar{x} = 7.7$  min,  $P < 0.05$ , Mann-Whitney  $U$ ). During focal observations 19 coalitions were formed between a subject and another male during a fight with a third male (Table 1). Of the 10 different subjects involved in a coalition, 9 more frequently aided a brother than a non-brother ( $P < 0.05$ , sign test). Agonistic coalitions have been reported for several macaque species<sup>6-8</sup> and probably have a significant effect on the success of brothers in non-natal groups. This hypothesis is supported by the dominance rank history of the sibling triad G6, N6 and 8G. The oldest brother disappeared during a food shortage and several weeks later the middle brother (N6) was attacked and died from infection. The youngest brother (8G) immediately dropped four positions in the male dominance hierarchy, the only male to decrease more than one rank-position during the study.

When an oestrous female was soliciting or consorting with a male, she was often displaced from that male (or attacked) by another adult male ( $n = 156$  disruptions, Table 2). For 13 of 15 subjects, the observed number of disruptions involving brothers was less than the expected value based on males randomly receiving or sending disruptions ( $P < 0.05$ , sign test). These data suggest that brothers avoided disrupting each other's sexual interactions.

During the colony's history, brothers who had been in the same group ( $n = 26$ ) accrued more months in those groups ( $\bar{x} = 31.7$ ) than did the 25 males who were not in their brothers' groups ( $\bar{x} = 21.4$ ,  $P < 0.05$ , Mann-Whitney  $U$ ). This difference was not due to age, as the males who did not join their brothers' groups were older ( $\bar{x} = 8.7$  yr) than those who did join their brothers ( $\bar{x} = 8.1$  yr). Nor was the difference due to the maternally dependent ranks of the males<sup>9</sup>. Those from the top-ranking half of their natal group did not spend significantly more time in non-natal groups than those from the bottom-ranking half ( $\bar{x} = 26.2$  and 27.9 months, respectively).

As a male accrues time within a non-natal group, he increases not only the number of breeding seasons during which he can

**Table 2** Disruptions of interactions with oestrous females involving brothers

| Subject | Total disruptions | P     | Disruptions involving brother |              | Sign |
|---------|-------------------|-------|-------------------------------|--------------|------|
|         |                   |       | Expected (E)                  | Observed (O) |      |
| 9I      | 13                | 0.071 | 0.94                          | 0            | —    |
| 5C      | 8                 | 0.071 | 0.57                          | 0            | —    |
| E4      | 8                 | 0.308 | 2.46                          | 1            | —    |
| O8      | 5                 | 0.308 | 1.54                          | 1            | —    |
| 1D      | 9                 | 0.308 | 2.77                          | 2            | —    |
| 7P      | 19                | 0.308 | 5.85                          | 5            | —    |
| 2T      | 10                | 0.308 | 3.08                          | 3            | —    |
| 7Z      | 6                 | 0.091 | 0.55                          | 1            | +    |
| CV7     | 2                 | 0.091 | 0.18                          | 0            | —    |
| AC9     | 9                 | 0.091 | 0.82                          | 0            | —    |
| CT5     | 7                 | 0.091 | 0.64                          | 1            | +    |
| 1L      | 12                | 0.067 | 0.80                          | 0            | —    |
| 7S      | 21                | 0.067 | 1.41                          | 1            | —    |
| X0      | 12                | 0.067 | 0.80                          | 0            | —    |
| 2L      | 15                | 0.067 | 1.01                          | 0            | —    |

Probability ( $P$ ) of a brother sending or receiving a disruption = (no. of brothers subject has in group) ÷ (no. of reproductively active males in group - 1). Expected frequency of a disruption involving a brother equals the product of  $P$  and the total number of disruptions. Sign decision is based on whether observed brother disruptions are greater or less than expected number of brother disruptions ( $P < 0.05$ , sign test).

father offspring, but also his dominance rank<sup>1,10</sup>. As there is a positive association between dominance rank and RS<sup>11,12</sup>, a male who accrues time (and therefore rank) in a group probably sires, on the average, relatively more offspring each breeding season (ref. 10 and unpublished data).

Affiliations among brothers have been described briefly for several nonhuman primates<sup>13,14</sup>. One possible mechanism by which brothers develop affiliative behaviours is attachment<sup>15</sup>, which may result from their close association with their mother. This hypothesis is supported by the fact that the average age difference between brothers in the same groups was 1.7 yr, while that between brothers in different groups was 2.6 yr. Other data, however, suggest that monkeys raised in isolation from birth associate preferentially with unfamiliar siblings<sup>16</sup>. Hence, both kin recognition and attachment may result in nepotism. Whatever the mechanisms which result in affiliative behaviours among brothers, our data are consistent with the hypothesis that nepotistic brothers have high inclusive fitness, and Hamilton's model<sup>17</sup> remains a plausible explanation for the evolution of nepotism among rhesus monkey brothers.

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- Drickamer, L. C. & Vessey, S. H. *Primates* **14**, 359-368 (1973).
- Lindburg, D. G. *Science* **166**, 1176-1178 (1969).
- Altmann, J. *Behaviour* **49**, 227-265 (1974).
- Vandenbergh, J. G. *Behaviour* **29**, 179-194 (1967).
- Vessey, S. H. *Folia Primatol.* **8**, 228-239 (1968).
- Kurland, J. *Contr. Primatol.* **12**, (1977).
- Massey, A. *Behav. Ecol. Sociobiol.* **2**, 31-40 (1977).
- Kaplan, J. *Am. J. phys. Anthropol.* **49**, 241-250 (1978).
- Sade, D. S. in *Social Communication Among Primates* (ed. Altmann, S.) 99-114 (University of Chicago Press, 1967).
- Bernstein, I. S. *J. theor. Biol.* **60**, 459-472 (1976).
- Duvall, S. W., Bernstein, I. S. & Gordon, T. P. *J. Reprod. Fert.* **47**, 25-31 (1976).
- Smith, D. G. *Am. J. phys. Anthropol.* **53**, 243-249 (1980).
- Goodall, J. in *Primate Behavior: Field Studies of Monkeys and Apes* (ed. DeVore, I.) 425-481 (Holt, Rinehart & Winston, New York, 1965).
- Boelkins, R. C. & Wilson, A. P. *Primates* **13**, 125-140 (1972).
- Cairns, R. B. *Psychol. Rev.* **73**, 409-426 (1966).
- Wu, H. M. H., Holmes, W. G., Medina, S. R. & Sackett, G. P. *Nature* **285**, 225-227 (1980).
- Hamilton, W. D. *J. theor. Biol.* **7**, 1-52 (1964).

**Table 1** Numbers of coalitions observed between the subjects and their brothers or non-brothers

| Subject | Coalition between: |              | Sign |
|---------|--------------------|--------------|------|
|         | Brothers           | Non-brothers |      |
| 9I      | 1                  | 0            | +    |
| E4      | 1                  | 0            | +    |
| 1D      | 1                  | 0            | +    |
| 7Z      | 3                  | 0            | +    |
| CV7     | 3                  | 1            | +    |
| AC9     | 1                  | 0            | +    |
| CT5     | 2                  | 0            | +    |
| 1L      | 0                  | 1            | —    |
| 7S      | 2                  | 0            | +    |
| G6      | 1                  | 0            | +    |

Nine of the 10 subjects were more frequently involved in a coalition with a brother than with a non-brother ( $P < 0.05$ , sign test).

## Opiates and clonidine prolong calcium-dependent after-hyperpolarizations

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& Alan North\*

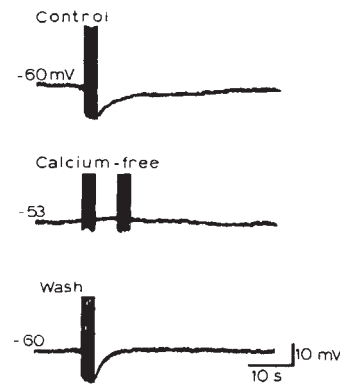
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Opiates<sup>1,2</sup> and  $\alpha$ -agonists<sup>3,4</sup> bind to separate and structurally specific sites on neurones in the central<sup>1,3</sup> and enteric nervous systems<sup>2,4</sup>. One functional consequence of this binding is inhibition of cell firing<sup>5,6</sup>, which may be due to hyperpolarization of the resting membrane by opiates, such as has been observed in the guinea pig locus coeruleus<sup>7</sup> and myenteric plexus<sup>8</sup>, and by clonidine in the myenteric plexus<sup>9</sup>. However, the discharge frequency of many nerve cells is limited by the membrane hyperpolarization which follows a period of activity, and which is caused by a transient increase in the intracellular calcium concentration leading to the activation of a membrane potassium conductance<sup>10</sup>. Neurones of the guinea pig myenteric plexus exhibit such a calcium-dependent potassium conductance<sup>11-13</sup>. We now report that both opiates and clonidine prolong this calcium-dependent after-hyperpolarization at concentrations (100 pM–10 nM) which are considerably lower than those usually required to hyperpolarize the resting membrane. Such a prolongation of the after-hyperpolarization will limit the frequency of discharge of neurones without altering their resting potential. The nature of the effects of morphine and clonidine are of interest in view of the similarities between the anatomical distribution of binding sites for these two drugs and the close parallels between their pharmacological effects in animals and man.

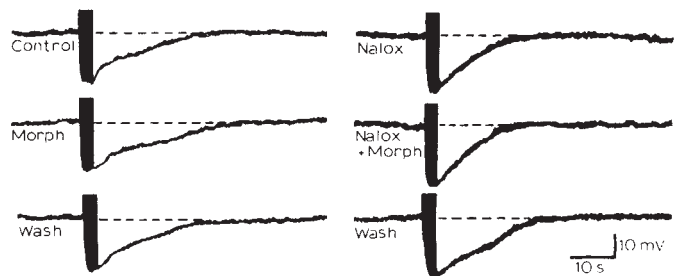
Intracellular recordings were made for more than 60 neurones in myenteric ganglia isolated from guinea pigs<sup>12</sup>. The neurones were bathed in a physiological saline solution (see Fig. 1 legend) to which morphine, clonidine or other drugs could be added. Action potentials were evoked by passing brief depolarizing currents through the recording electrode. Most of the experiments were on neurones which showed long-lasting after-hyperpolarization following a single action potential (type 2 (ref. 12) or AH cells<sup>13</sup>). Type 1 cells<sup>12</sup> or S cells<sup>13</sup> only showed an after-hyperpolarization following a train of action potentials. The after-hyperpolarization following a train of action potentials (usually 10 Hz for 3 s) was completely eliminated by cobalt (3 mM) or by perfusion with calcium-free solution (Fig. 1).

Morphine (100 pM–10 nM) prolonged the after-hyperpolarization which followed a train of action potentials. The effect of morphine was concentration dependent in the range tested, and reversed within 5–10 min of washing with morphine-free solution (Fig. 2). Naloxone (1 nM) reversibly abolished the action of morphine. The prolongation occurred without marked change in amplitude or time course of the initial component of the after-hyperpolarization (see Fig. 2 legend), suggesting that morphine may inhibit the mechanism by which a neurone normalizes its free intracellular calcium concentration following the action potentials.

Doubling the extracellular calcium concentration or the application of tetraethylammonium bromide (1 mM), increased the duration of the after-hyperpolarization. Morphine was much less effective in further prolonging the after-hyperpolarization



**Fig. 1** Abolition of the hyperpolarization following a train of action potentials by removal of extracellular calcium ions. This and other figures are intracellular recordings of membrane potential. Depolarizing current pulses of sufficient amplitude to evoke action potentials were passed across the cell membrane (full spike amplitude is not shown). Each train consisted of 15 pulses at 5 Hz. Top trace, control recording. Resting potential,  $-60$  mV. Middle trace, 20 min after changing to a solution containing no calcium and 5 mM magnesium. The cell membrane depolarized by 7 mV, but even two trains of action potentials failed to elicit any after-hyperpolarization. Bottom trace, 20 min after returning to the control solution. All intracellular recordings were done using techniques described elsewhere<sup>12</sup>. Control perfusion solutions contained (mM): NaCl, 117; KCl, 4.7;  $\text{CaCl}_2$ , 2.54; MgCl, 1.2;  $\text{NaH}_2\text{PO}_4$ , 1.2;  $\text{NaHCO}_3$ , 25; glucose, 11, and were gassed with 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ .

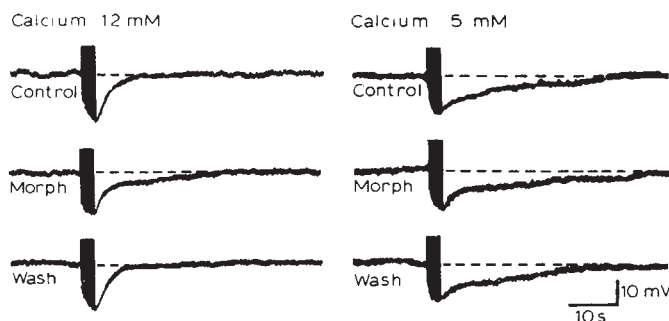


**Fig. 2** Prolongation of the after-hyperpolarization by morphine (1 nM). Left: top trace, control recording; middle trace, 2 min after commencing perfusion with a solution containing morphine sulphate (1 nM); bottom trace, 10 min after washing with a morphine-free solution. Right: top trace obtained after several minutes in naloxone hydrochloride (1 nM); middle trace, 2 min after changing to a solution which contained naloxone and morphine (1 nM); bottom trace, 10 min after washing with control solution. Morphine had no effect in the presence of naloxone; in this and other experiments, naloxone itself slightly reduced the after-hyperpolarization. Maximum amplitude of after-hyperpolarization was not affected by morphine (1 nM): control,  $17.5 \pm 0.70$  mV; morphine,  $18.4 \pm 0.53$  mV (mean  $\pm$  s.e., paired  $t$ -test,  $P > 0.05$ ,  $n = 11$ ). Time for decay to 50% ( $t_{50}$ ) or to 80% ( $t_{80}$ ) of peak amplitude was prolonged by morphine (1 nM): control,  $t_{50} = 12.2 \pm 1.5$  s; morphine,  $t_{50} = 16.0 \pm 2.0$  s (paired  $t$ -test,  $P < 0.01$ ,  $n = 11$ ). Control,  $t_{80} = 24.5 \pm 3.0$  s; morphine,  $t_{80} = 36.0 \pm 4.3$  s (paired  $t$ -test,  $P < 0.001$ ,  $n = 11$ ).

in the presence of an elevated (5.08 mM) calcium ion concentration (Fig. 3). Reducing the calcium ion concentration to one-half (1.26 mM) its control value decreased the duration of the after-hyperpolarization; in this case the effect of morphine became greater (Fig. 3). The lower calcium concentration may be much closer to that of 'free' calcium in the extracellular space of the nervous system<sup>14</sup>.

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**Fig. 3** Effect of morphine in high and low calcium concentrations. Left, recordings from the same neurone shown in Fig. 2, but after 30 min in a solution containing low (1.2 mM) calcium concentration. Top trace, control; middle trace, after 2 min perfusion with morphine (1 nM); bottom trace, wash. Right, further recordings from the same neurone, after 30 min in high (5 mM) calcium solution. Top trace, control; middle trace, after 1.5 min perfusion with morphine (1 nM); bottom trace, wash. The prolongation of the after-hyperpolarization by morphine was much more apparent when the after-hyperpolarization was shortened by reducing the calcium concentration.

Opiates inhibit the binding of calcium to brain synaptosomal membranes and the extent of this inhibition is reduced in high calcium solutions<sup>15</sup>. We speculate that occupation of the opiate receptor of myenteric neurones may lead to an inhibition of the binding of calcium to an intracellular site, perhaps on the inner surface of the membrane. If this site is normally important in the sequestration of calcium which enters the neurone during activity, such an inhibition would prolong the period of increased intracellular calcium and, therefore, the after-hyperpolarization. Such a mechanism could also underlie the hyperpolarization of the resting membrane observed with higher morphine concentrations<sup>8</sup>.

Clonidine (100 pM–10 nM) prolonged the after-hyperpolarization in a similar manner to morphine, although a larger proportion of neurones also showed a hyperpolarization of the resting membrane. This action of clonidine was prevented by phentolamine (100 nM). The similarity between the acute effects of clonidine and morphine may underlie the closeness of the pharmacological profiles of the two drugs. It is further possible that their identical mechanism of action offers an explanation for the efficacy of clonidine in ameliorating the signs and symptoms of opiate withdrawal<sup>6,16</sup>.

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- Pert, C. B. & Snyder, S. H. *Science* **179**, 1011–1013 (1973).
- Leslie, F. M., Chavkin, C. & Cox, B. M. *J. Pharmac. exp. Ther.* **214**, 395–402 (1980).
- U'Prichard, D. C. & Snyder, S. H. *J. biol. Chem.* **252**, 6450–6463 (1977).
- Tanaka, T. & Starke, K. *Naunyn-Schmiedeberg's Archs Pharmac.* **309**, 207–215 (1979).
- North, R. A. *Life Sci.* **24**, 1527–1546 (1979).
- Aghajanian, G. K. *Nature* **276**, 186–188 (1978).
- Pepper, C. M. & Henderson, G. *Science* **209**, 394–396 (1980).
- North, R. A. & Tonini, M. *Br. J. Pharmac.* **61**, 541–549 (1977).
- Morita, K. & North, R. A. *Br. J. Pharmac.* **74**, 419–428 (1981).
- Meech, R. W. A. *Rev. Biophys. Bioengng.* **7**, 1–18 (1978).
- North, R. A. *Br. J. Pharmac.* **49**, 708–711 (1973).
- Nishi, S. & North, R. A. *J. Physiol., Lond.* **231**, 471–491 (1973).
- Hirst, G. D. S., Holman, M. E. & Spence, I. *J. Physiol., Lond.* **236**, 303–326 (1974).
- Nicholson, C., ten Bruggencate, G., Stockle, H. & Steinberg, R. *J. Neurophysiol.* **41**, 1026–1039 (1978).
- Guerrero-Munoz, F., Cerrata, K. V., Guerrero, M. L. & Way, E. L. *J. Pharmac. exp. Ther.* **209**, 132–136 (1978).
- Gold, M. S., Redmond, D. E. & Kleber, H. D. *Lancet* **ii**, 599–602 (1978).

## Calmodulin localization during capping and receptor-mediated endocytosis

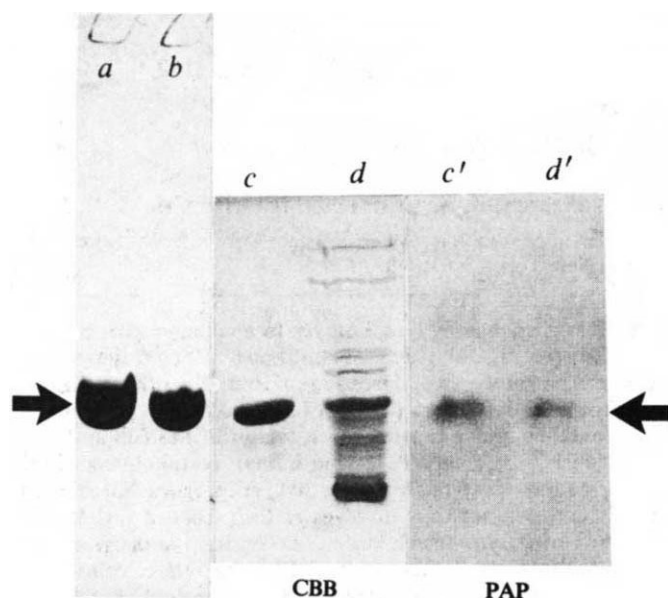
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The early response by lymphocytes to challenge with cell surface-directed ligand, such as antibody or concanavalin A (Con A), involves the clustering of initially diffuse ligand-receptor complexes into patches, followed by the gathering of these patches into a cap over one region of the cell and their endocytosis<sup>1–3</sup>. Using cells of the human lymphoblastoid line WiL2, we have previously shown that receptor-mediated endocytosis occurring at caps involves clathrin-coated vesicles and that the formation of such vesicles is sensitive to drugs such as trifluoperazine dihydrochloride (TFP) that affect calmodulin (CaM), the calcium-dependent regulatory protein of cells<sup>3</sup>. By indirect immunofluorescence, we demonstrate here that in WiL2, before surface-directed ligand challenge, CaM is distributed diffusely in the cell. On challenge, concurrent with capping of cell-surface receptors for Con A, CaM localization becomes concentrated in regions of the cytoplasm just below the cap. We show that CaM redistribution is sensitive to TFP and dependent on  $\text{Ca}^{2+}$  in the external milieu. Capping is mostly unaffected by TFP or removal of external  $\text{Ca}^{2+}$ . Cytochalasin D (CD), on the other hand, blocks capping completely, but does not prevent the initial redistribution of CaM. These results are consistent with a functional role for CaM in clathrin recruitment to the cell surface beneath ligand-receptor complexes and an initial  $\text{Ca}^{2+}$  requiring, microfilament-independent event in receptor-mediated endocytosis.

Calmodulin has been implicated in the mediation of various cellular activities thought to involve transient fluxes of  $\text{Ca}^{2+}$  as signals<sup>4,5</sup>. In particular, CaM– $\text{Ca}^{2+}$  has been shown to regulate myosin light chain kinase of smooth muscle and non-muscle cells<sup>6–10</sup> and may be an important intermediate in the  $\text{Ca}^{2+}$  control of microfilament-based motility in such cells. Immunofluorescence techniques have been used to visualize the localization of CaM in a variety of cells<sup>11–17</sup>. To study the intracellular distribution of CaM in WiL2 cells during Con A capping and endocytosis, we prepared rabbit antiserum against purified bovine brain CaM (Fig. 1a, b) as outlined by Wallace and Cheung<sup>18</sup>. This antiserum specifically identifies one polypeptide band, CaM, in a crude brain extract by a gel transfer peroxidase–antiperoxidase staining technique<sup>19</sup> (see Fig. 1). The antiserum has also been used in preliminary experiments specifically to immunoprecipitate WiL2 CaM metabolically labelled with <sup>35</sup>S-methionine (J. L. Salisbury and R. Green, unpublished observations). We further purified the anti-CaM IgG by affinity chromatography on a CaM–Sepharose column according to Dedman and co-workers<sup>11</sup> (see Fig. 1 legend) and, using the purified immunoglobulin as the primary antibody, we followed the reorganization of intracellular CaM during Con A receptor capping using double labelling techniques (Fig. 2). In these studies Con A receptors and CaM could be followed in the same cell using Con A covalently labelled with fluorescein isothiocyanate (FITC–Con A), which fluoresces yellow-green, and anti-CaM labelled with a secondary antibody, rhodamine-conjugated goat anti-rabbit IgG, which fluoresces red.

CaM localization during capping appears concentrated at sites of endocytosis. When WiL2 cells are fixed immediately after ligand challenge and labelled with anti-CaM (as described in Fig. 2g–i legend) they exhibit a diffuse cell-surface FITC–Con A localization (Fig. 2h) and a diffuse intracellular distribution of rhodamine-labelled anti-CaM (Fig. 2i) which is brighter than the diffuse low level of rhodamine fluorescence displayed by

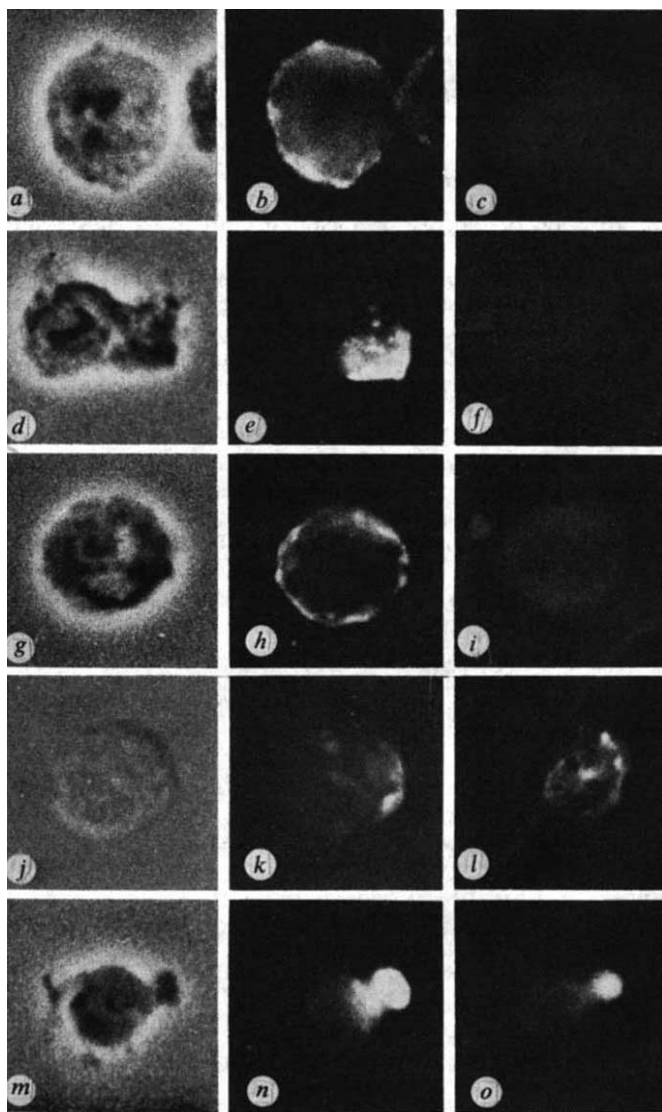


**Fig. 1** Rabbit antiserum against bovine brain CaM was prepared according to Wallace and Cheung<sup>18</sup>. Track *a* is a SDS-polyacrylamide electropherogram showing a sample of the highly purified bovine brain CaM used to prepare the dinitrophenyl (DNP)-CaM shown in track *b*, which was subsequently used as the antigen. The antisera contained non-precipitating anti-CaM antibodies which were detected by the peroxidase-antiperoxidase staining of anti-CaM serum-treated nitrocellulose transfers from 15% polyacrylamide microslab gels containing 0.1% SDS into which either purified (tracks *c*, *c'*) or heat-treated crude CaM containing extracts from bovine brain (tracks *d*, *d'*) were electrophoresed. Shown here is a Coomassie brilliant blue stained gel (CBB) and a peroxidase-antiperoxidase (PAP) stained transfer of a companion gel. CaM is the band near the centre of each track (arrows). Specific anti-CaM antibody was affinity purified from the IgG fraction of immunized rabbit antiserum by adsorption on to native CaM covalently linked to Sepharose 4B. Following an extensive wash, specifically bound anti-CaM IgG was eluted with a pulse of 0.2 M glycine pH 2.8, adjusted to pH 7.5 with 1 M phosphate buffer and dialysed against phosphate-buffered saline (PBS).

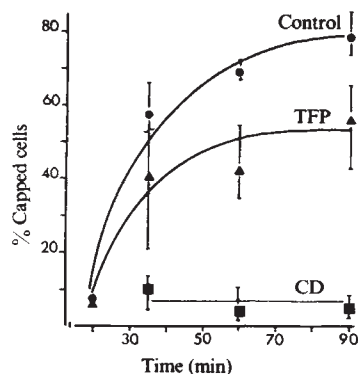
**Fig. 2** Indirect immunofluorescent localization of CaM during Con A receptor capping. Indirect immunofluorescent localization of anti-CaM binding sites was carried out using the affinity purified anti-CaM and rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories). WiL2 cells were challenged with FITC-Con A ( $10 \mu\text{g ml}^{-1}$ ) to induce capping of Con A receptors. At various times after FITC-Con A challenge the cells were briefly fixed with formaldehyde, extracted in  $-20^\circ\text{C}$  absolute acetone, reacted with affinity purified anti-CaM, washed with PBS and reacted with rhodamine-conjugated goat anti-rabbit IgG. After washing, the cells were observed using a Zeiss photomicroscope equipped for UV epi-illumination. The cell-surface distribution of FITC-Con A and the intracellular localization of rhodamine-labelled anti-CaM could be separately visualized on or in the same cell by switching to the appropriate excitation filter, beam splitter and barrier filter (Zeiss BP 450-490, FT 510 and BP 520-560 for FITC and BP 456/10, FT 580, LP 590 for rhodamine). *a-c*, WiL2 cell challenged with FITC-Con A, immediately fixed and treated with preimmune antiserum followed by rhodamine-labelled goat anti-rabbit IgG showing the diffuse ring-like surface localization of FITC-Con A receptors (*b*) and the control level of background rhodamine fluorescence (*c*). The same level of background fluorescence was observed when the primary antibody was omitted entirely or when anti-CaM was first preabsorbed with excess CaM. *d-f*, WiL2 cell treated as in *a-c* but first allowed to cap the FITC-Con A receptors (*e*) at  $37^\circ\text{C}$  before fixation, again illustrating the control level of background rhodamine fluorescence (*f*). *g-i*, WiL2 cell challenged with FITC-Con A, immediately fixed and treated with the affinity purified anti-CaM IgG followed by rhodamine-labelled goat anti-rabbit IgG. The FITC-Con A is diffusely located at the cell surface (*h*) while the rhodamine-labelled anti-CaM is diffuse throughout the cytoplasm (*i*). *j, k*, WiL2 cell treated as in *g-i* but first allowed to begin capping the FITC-Con A receptors at  $37^\circ\text{C}$  for 30 min before fixation. Note the lateral redistribution of FITC-Con A receptors at the cell surface (*k*) accompanied by a dramatic reorganization of the intracellular anti-CaM binding sites beneath the capping FITC-Con A (*l*). *m-o*, WiL2 cell treated as in *g-i* but first allowed to completely cap the FITC-Con A for 1 h before fixation. Here the rhodamine-labelled anti-CaM has reorganized within the cytoplasm (*o*) beneath the tight FITC-Con A cap (*n*). The same pattern of specific anti-CaM localization was observed when whole immune serum was substituted for affinity purified serum. Each row, left to right, shows the same cell. *a, d, g, j, m*, Light micrograph; *b, e, h, k, n*, FITC-Con A fluorescence; *c, f, i, l, o*, rhodamine fluorescence. All  $\sim \times 1,460$ . Fluorescence micrographs were recorded using Tri-X (Kodak) film and developed in D19 for 5 min.

control cells labelled with preimmune serum (Fig. 2c). We interpret this to indicate that CaM is uniformly distributed throughout the unstimulated cell in which Con A receptors are diffuse and that receptor-mediated endocytosis is occurring only at a basal rate. When the cells are allowed to cap the FITC-Con A at  $37^\circ\text{C}$  for 30 min before fixation (Fig. 2j-l) a dramatic redistribution of intracellular anti-CaM binding sites occurs. As FITC-Con A receptors begin to move laterally towards one region of the cell surface (Fig. 2k), the originally diffuse anti-CaM binding sites also move to regions in the cytoplasm just beneath the large clustered regions of Con A and sometimes also appear at a focal region near the cell centre (Fig. 2l). Control cells allowed to cap FITC-Con A (Fig. 2e) and then treated either with preimmune serum instead of anti-CaM, omitting primary antibody, or by preabsorbing anti-CaM with excess CaM, still show only diffuse low-level rhodamine staining (Fig. 2f).

When cells are allowed to cap the FITC-Con A completely (Fig. 2n), rhodamine-labelled anti-CaM binding sites become further reorganized in the cytoplasm beneath the cap (Fig. 2o). This suggests that after ligand challenge, CaM is bound to and moves with some cytoplasmic structure that itself co-caps with the ligand. As indirect immunofluorescence studies<sup>19</sup> indicate that actin and myosin co-cap with certain surface receptors and co-isolate with Con A caps from *Dictyostelium*<sup>20</sup>, one potential CaM binding structure is the microfilament network of the cell cortex. Alternatively, the finding that endocytosis accompanies capping and is sensitive to CaM-directed drugs<sup>3</sup> and that CaM co-isolates with coated vesicles from WiL2 cells<sup>3</sup>, suggests that a







**Fig. 3** Kinetics of capping after challenge with Con A and the effects of cytochalasin D (CD) and trifluoperazine (TFP) on capping. WiL2 cells were incubated in PBS (●), cytochalasin D (Sigma),  $10 \mu\text{g ml}^{-1}$  (■), or trifluoperazine (Smith Kline & French),  $15 \mu\text{M}$  (▲), for 10 min, challenged with  $10 \mu\text{g ml}^{-1}$  FITC-Con A and allowed to cap the receptor-ligand complexes at  $37^\circ\text{C}$ . At various times after ligand challenge, cells were fixed and fields of 20–30 cells photographed using the fluorescence microscope. Each point represents the mean value of % capped cells for a minimum of 100 cells, the bars represent the maximum range for individual fields. A capped cell is defined here as one which has redistributed the FITC-Con A, clearing at least half of its cell surface of label.

second potential CaM binding site is the membrane of clathrin-coated pits and vesicles. We have investigated these suggestions in a series of experiments using drug inhibitors of capping and endocytosis.

We have previously reported<sup>3</sup> that the cytochalasin derivative dihydrocytochalasin B inhibits capping of cell-surface IgM by WiL2 cells. However, it has no apparent effect on the recruitment of clathrin coat material to ligand-IgM complexes and only partially reduces endocytosis of the complexes<sup>3</sup>. Similarly, although CD ( $10 \mu\text{g ml}^{-1}$ ) inhibits Con A capping almost completely (Fig. 3), this drug does not inhibit eventual endocytosis of ligand (to be published elsewhere).

Trifluoperazine dihydrochloride at low concentration ( $10$ – $25 \mu\text{M}$ ) is a potent and highly specific inhibitor of CaM-mediated processes<sup>21–24</sup>, and binds to CaM with high affinity in the presence of  $\text{Ca}^{2+}$  (refs 22, 23). It inhibits CaM stimulation of brain phosphodiesterase<sup>22,23</sup> and adenylate cyclase<sup>23</sup>, inactivates CaM regulation of smooth muscle contraction<sup>24</sup>, and in WiL2 cells, significantly blocks endocytosis of surface receptors<sup>3</sup>. An important effect of TFP is the inhibition of clathrin recruitment to clustered receptor complexes, which suggests that clathrin recruitment is a CaM-regulated event<sup>3</sup>. TFP does not prevent capping of either Con A receptors (Fig. 3) or receptor-IgM in WiL2 cells<sup>3</sup>, in contrast to its effect in other cells<sup>25</sup>, but it does reduce the final percentage of tightly capped cells by about one-third (Fig. 3).

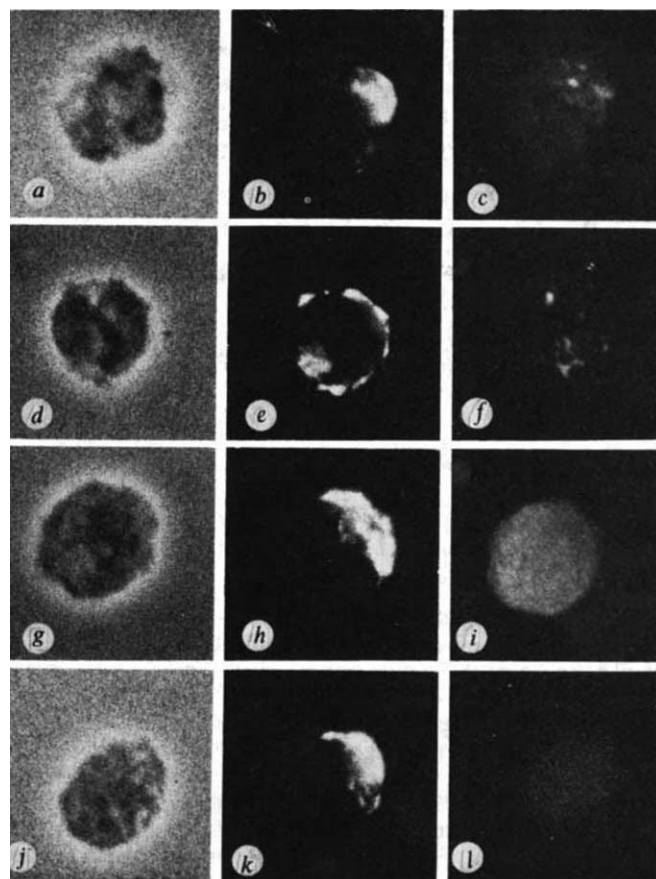
The differential effects of CD and TFP on receptor capping have allowed us to explore whether CaM redistribution on ligand challenge is obligately coupled to capping. Figure 4a–c illustrates the reorganization of intracellular CaM during capping by WiL2 cells in normal conditions and corresponds to Fig. 2j–l. When WiL2 cells are incubated with CD ( $10 \mu\text{g ml}^{-1}$ ) but otherwise treated as in Fig. 4a–c, Con A receptor capping is inhibited (Figs 3, 4e). The FITC-Con A is patched and by 30 min is mostly still at the cell surface although it is eventually internalized<sup>3</sup>. Anti-CaM binding sites are not diffuse in the CD-treated cells. Rather, rhodamine-labelled anti-CaM appears as numerous 'hot spots' in the cytoplasm (Fig. 4f), many of which lie directly beneath patches of FITC-Con A at the cell surface (Fig. 4e, f). In the absence of ligand challenge, CD treatment does not result in CaM redistribution (not shown). Therefore, CaM reorganization follows ligand challenge and does not depend on receptor capping. Receptor capping and CaM redistribution are separable events.

When WiL2 cells are incubated with TFP ( $15 \mu\text{M}$ ) and treated as in Fig. 4a–c, Con A receptor capping still occurs (Fig. 4h) in most of the cells, albeit in a lower percentage than the controls (see Fig. 3). Nevertheless, in these TFP-treated capped cells

anti-CaM staining is diffuse throughout the cell (Fig. 4i). The rhodamine-labelled anti-CaM is considerably brighter in TFP-treated cells than in any other treatment. We do not know why this is so; in these conditions TFP autofluorescence is undetectable. Perhaps TFP binding to CaM in the cytoplasm unmasks antigenic determinants on the molecule. In any event, these results clearly show that capping can occur in the presence of TFP, independently of CaM redistribution.

To probe the effect of  $\text{Ca}^{2+}$  on these processes, we have begun chelator experiments. When WiL2 cells are incubated in conditions where the external free  $\text{Ca}^{2+}$  concentration is held below  $10^{-7} \text{M}$  by inclusion of the calcium chelator EGTA in the external medium, capping of Con A receptors proceeds normally (Fig. 4k), in agreement with the findings of other workers<sup>2</sup>. In these conditions anti-CaM binding sites again remain diffuse (Fig. 4l). These results suggest that  $\text{Ca}^{2+}$  is a necessary intermediate in the coupling of capping and CaM redistribution.

The observations reported here, taken with our earlier studies<sup>3</sup>, support the suggestion that receptor-mediated endocytosis operates under the control of CaM- $\text{Ca}^{2+}$  signals that involve transient fluxes of  $\text{Ca}^{2+}$  across the plasma membrane at sites of ligand-receptor clusters. In our experiments the structural redistribution of CaM after ligand challenge and presumably CaM- $\text{Ca}^{2+}$  complex formation corresponds to the formation and distribution of coated endocytotic vesicles rather than to microfilaments. In this regard, it is important to recall that CaM appears to be an intrinsic component of coated vesicles isolated from WiL2 cells<sup>3</sup> and from brain<sup>26</sup>. Although in normal conditions coated vesicle formation is closely coupled to microfilament formation and function, this coupling is not



**Fig. 4** Receptor capping and CaM redistribution are separable events. WiL2 cells were incubated in PBS (a–c), cytochalasin D,  $10 \mu\text{g ml}^{-1}$  (d–f), trifluoperazine,  $15 \mu\text{M}$  (g–i) or EGTA,  $5 \text{mM}$  (j–l) for 10 min, challenged with  $10 \mu\text{g ml}^{-1}$  FITC-Con A and allowed to cap the receptor-ligand complexes for 30 min at  $37^\circ\text{C}$ . Cells were fixed and then processed for anti-CaM staining as described in Fig. 2 legend. Each row, left to right, shows the same cell. a, d, g, j, Phase micrographs; b, e, h, k, FITC-Con A fluorescence; c, f, i, l, anti-CaM rhodamine fluorescence. All  $\sim \times 1,460$ .

obligatory. Two sites of action of  $\text{CaM-Ca}^{2+}$  are interesting potential control points in this process: (1) The activation of myosin light chain kinase for acceleration of capping. This might explain the partial inhibition of capping seen on TFP treatment. (2) The recruitment of clathrin coats to the membrane.

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1. Taylor, R. B., Duffus, W., Raff, M. & dePetris, S. *Nature new Biol.* **233**, 225–229 (1971).
2. Bhalla, D. K., Hunt, C. V., Kapur, S. P. & Anderson, W. A. *J. Cell Sci.* **36**, 31–44 (1979).
3. Salisbury, J. L., Condeelis, J. S. & Satir, P. *J. Cell Biol.* **87**, 132–141 (1980); *Ann. N.Y. Acad. Sci.* **356**, 429–432 (1980).
4. Cheung, W. Y. *Science* **207**, 19–27 (1980).
5. Wang, J. H. & Waisman, D. M. *Curr. Topics cell. Regulation* **15**, 47–107 (1979).
6. Dabrowska, R., Sherry, J. M. F., Aromatorio, D. & Hartshorne, D. J. *Biochemistry* **17**, 253–258 (1978).
7. Scholey, J. M., Taylor, K. A. & Kendrick-Jones, J. *Nature* **287**, 233–235 (1980).
8. Yerna, M.-J., Dabrowska, R., Hartshorne, D. J. & Goldman, R. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 184–188 (1979).
9. Nishikawa, M., Tanaka, T. & Hidaka, H. *Nature* **287**, 863–865 (1980).
10. Walsh, M. P., Vallet, B., Cavadore, J.-C. & Demaille, J. G. *J. biol. Chem.* **255**, 335–337 (1980).
11. Dedman, J. R., Welsh, M. J. & Means, A. R. *J. biol. Chem.* **253**, 7515–7521 (1978).
12. Andersen, B., Osborn, M. & Weber, K. *Cytobiologie* **17**, 354–364 (1978).
13. Welsh, M. J., Dedman, J. R., Brinkley, B. R. & Means, A. R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1867–1871 (1978).
14. Mailhe, N. J. & Satir, B. *J. Protozool.* **26**, 10a (1979); *Ann. N.Y. Acad. Sci.* **356**, 408–412 (1980).
15. Mailhe, N. J., Dedman, J. R., Means, A. R., Chafouleas, J. G. & Satir, B. H. *J. Cell Biol.* **89**, 695–699 (1981).
16. Jones, H. P., Lenz, R. W., Palevitz, B. A. & Cormier, M. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2772–2776 (1980).
17. Glenney, J. R. Jr & Weber, K. *J. biol. Chem.* **255**, 10551–10554 (1980).
18. Wallace, R. W. & Cheung, W. Y. *J. biol. Chem.* **254**, 6564–6571 (1980).
19. Sternberger, L. *Mikroskopie* **25**, 346 (1969).
20. Bourguignon, L. Y. W., Tokuyasu, K. T. & Singer, S. J. *J. gen. Physiol.* **95**, 239–258 (1978).
21. Condeelis, J. S. *J. Cell Biol.* **80**, 751–758 (1979).
22. Levin, R. M. & Weiss, C. B. *Molec. Pharmacol.* **12**, 581–589 (1976); *J. Pharmac. exp. Ther.* **208**, 454–459 (1979).
23. Weiss, C. B. & Levin, R. M. *Adv. Cyclic Nucleotide Res.* **9**, 285–304 (1978).
24. Hidaka, H., Yamaki, T., Totsuka, T. R. & Asano, M. *Molec. Pharmacol.* **15**, 49–59 (1979).
25. Bourguignon, L. Y. W. & Balazovich, K. *Cell Biol. int. Rep.* **4**, 947–952 (1980).
26. Linden, C. D., Dedman, J. R., Chafouleas, J., Means, A. R. & Roth, T. F. *Proc. natn. Acad. Sci. U.S.A.* **78**, 308–312 (1981).

## Long-term human T-helper lines producing specific helper factor reactive to influenza virus

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Our understanding of the mechanism of T-cell help in antibody production in the mouse has been improved by the production of long-term lines of T cells, either using T-cell growth factor (TCGF or IL-2) or by creating T-cell hybridomas. Such cells have been shown to produce non-antigen-specific<sup>1,2</sup> or antigen-specific helper factors<sup>3</sup> with genetically restricted or unrestricted activity<sup>1–3</sup>. However, the factors are still not well characterized. We have examined the role of human T-helper cells and their soluble factors in inducing antibody production, initially in a xenogeneic system, using mouse B cells<sup>4</sup>. Using a human *in vitro* secondary antibody response system for influenza virus<sup>5</sup> we have investigated the effect of helper factors on human B cells. We report here the production of long-term cultures of antigen-specific human helper cells. Both the cells and the high-potency antigen-specific helper factor (HF) which they produce act on human B cells in a genetically restricted manner. Preliminary mapping of the restriction indicates associations, but not identity, with HLA-DR.

Table 1 Cell membrane phenotype of F<sub>7</sub> T-cell line

| Marker     | % Of positive cells |         |
|------------|---------------------|---------|
|            | Day 40              | Day 150 |
| UCHT1      | 93                  | 98      |
| OKT1       | ND                  | 98      |
| Leu 2a     | 0                   | 3       |
| Leu 3a     | 83                  | 82      |
| HLA-DR     | 72                  | 40      |
| OKM1       | ND                  | 2       |
| BA-1       | ND                  | 2       |
| S Ig       | 0                   | 0       |
| E rosettes | ND                  | >99     |

Human peripheral blood lymphocytes (PBLs) were prepared on a Ficoll-Hypaque gradient, then suspended at  $10^{-7}$  ml<sup>-1</sup> in RPMI 1640 containing 10% fetal calf serum (FCS) and mixed with two volumes of 8-(aminoethyl) isothiourea bromide hydrobromide-treated sheep red blood cells (SRBC). After incubation at 4°C, E<sup>+</sup> cells (T cell-enriched suspensions) were separated from non-rosetting cells (E<sup>-</sup>; T cell-depleted suspensions) by centrifugation through a Ficoll-Hypaque gradient. Red cells were removed by hypotonic shock. E<sup>+</sup> cells were cultured for 7 days at a concentration of  $1 \times 10^6$  ml<sup>-1</sup> with A/X31 (5 µg ml<sup>-1</sup>) in the presence of 10% horse serum in 50-ml Falcon flasks. Dead cells were then removed on a Ficoll-Hypaque gradient and the cells (containing 20–40% blasts) were re-cultured at a concentration of  $1 \times 10^5$  ml<sup>-1</sup> in RPMI + 10% FCS supplemented with TCGF medium diluted to an optimal concentration (~25%). The supernatants were prepared by culturing  $1 \times 10^6$  pooled human tonsil cells from different donors with 2 µg ml<sup>-1</sup> PHA in RPMI-5% human serum for 48 h. PHA was removed from TCGF medium by passage over a column of Sepharose 4B to which rabbit anti-PHA immunoglobulin had been coupled<sup>17</sup>. PHA-depleted medium was no longer able to induce the proliferation of PBLs. TCGF medium was filtered and stored at -20°C. Every 3–4 days, medium was removed by centrifugation and cells re-seeded at  $1 \times 10^5$  cells ml<sup>-1</sup> in fresh TCGF medium in the absence of antigen. F<sub>7</sub> T cells have been kept growing for >4 months. The average doubling time is 48 h. Aliquots of F<sub>7</sub> cells ( $2 \times 10^5$ ) were incubated for 30 min on ice with saturating concentrations, previously determined by titration, of monoclonal antibodies (culture supernatant or purified immunoglobulin) washed three times in HEPES-buffered RPMI medium with 5% FCS and stained for 30 min on ice with human immunoglobulin-adsorbed and immunoadsorbent-purified fluorescein isothiocyanate-conjugated (FITC) sheep anti-mouse immunoglobulin antiserum. After a further three washes the cells were either examined by fluorescence microscopy or analysed by Becton-Dickinson FACS I or IV. Controls and precautions to minimize non-specific binding have been described previously<sup>18</sup>. Monoclonal antibodies used were: UCHT1 (against all mature peripheral human T lymphocytes<sup>19</sup>); Leu 2a and 3a (gifts from Becton-Dickinson); anti-HLA-DR (DA2)<sup>19</sup> (gift from Dr M. Crumpton); OKM1 and OKT11 (gifts from Dr G. Goldstein); and BA-1 (ref. 6) gift from Dr J. Kersey. Polyspecific FITC sheep anti-human immunoglobulin antiserum was prepared in our laboratory—this brightly stains human peripheral blood B lymphocytes. ND, not determined.

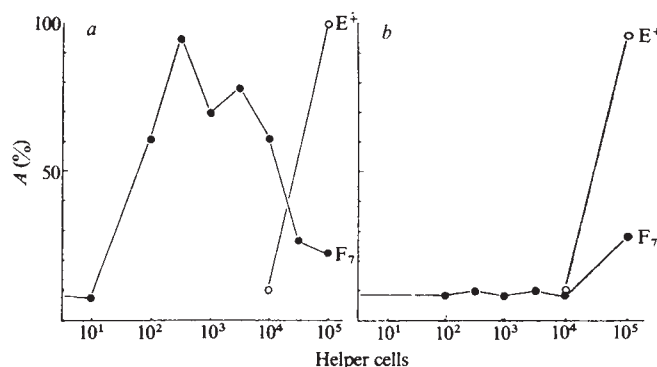
Peripheral blood lymphocytes from donors (laboratory volunteers) known to respond to strain A influenza virus (A/X31) and strain B, Hong Kong B virus (HKB), were cultured with A/X31 for 7 days before addition of human TCGF depleted of phytohaemagglutinin (PHA). Several lines have been obtained which, after 4–6 weeks of growth, displayed stable characteristics. The surface characteristics of one of these cell lines, F<sub>7</sub>, are shown in Table 1. After 6 weeks, essentially all the cells were T cells (UCHT1<sup>+</sup>, E<sup>+</sup>, and OKT11<sup>+</sup>, OKM1<sup>-</sup>) of the helper type Leu 3a<sup>+</sup>, 2a<sup>-</sup> (see Table 1). A high, but variable proportion of cells stained with antisera to HLA-DR throughout culture. No staining was seen with conventional anti-immunoglobulin antisera or the monoclonal anti-B lymphocyte serum BA-1 (ref. 6). Analysis of these lines earlier in their development revealed different results, in that some cells were Leu 3a<sup>-</sup>, 2a<sup>+</sup>, but these cells were lost spontaneously during culture.

The helper activity of the T-cell lines was assayed on autologous B cells and monocytes using erythrocyte rosette-depleted cells<sup>5</sup> in the absence of TCGF. In a typical experiment (Fig. 1) with the F<sub>7</sub> cell line, 65 days after initiation of culture, optimal help was obtained with  $5 \times 10^2$  cells, compared with  $10^3$  fresh T cells from the same donor (before culture). Moreover, the number of antigen-responding helper T cells was ~1:10–1:30, as measured by limiting dilution, which is in the range of that observed for a cloned helper T-cell line in the mouse<sup>2</sup>. Note that in this and most other experiments, excess helper line cells markedly inhibited the response. The helper property of the cell



line remained after 5 months of culture. The specificity of the helper line was demonstrated by the markedly diminished capacity to respond to HKB compared with uncultured T cells (Fig. 1). Specificity was verified by the lack of a response to varicella-zoster virus, and in 'bystander' experiments with mixtures of A/X31 and HKB (data not shown).

We investigated the mechanisms of help by the cell lines. The best studied—H<sub>2</sub> (a similar cell line from another donor) and F<sub>7</sub>—yield high potency supernatants. These could be obtained from T cells cultured alone without TCGF, but higher titres of HF were obtained when autologous irradiated E<sup>+</sup> cells plus antigen (A/X31) were added (Fig. 2). Again, maximum help was obtained at low percentages of HF (0.1–1%), whereas higher concentrations were inhibitory. This help was specific, as the response of HKB was not influenced. Supernatants produced in the presence of A/X31 also demonstrated no bystander effect, as previously noted for mouse TCGF lines<sup>7</sup>. (This effect is the induction by specific antigen of a nonspecific factor which



**Fig. 1** Helper activity effected by A/X31-specific F<sub>7</sub> T-cell line. The method described by Callard<sup>5</sup> and modified by Zanders *et al.*<sup>20</sup> was used to assay the ability of washed TCGF-free F<sub>7</sub> cells to help E<sup>+</sup> cells in producing antibodies to A/X31. E<sup>+</sup> and E<sup>+</sup> cells were separated by centrifugation through a Percoll (Pharmacia)  $d = 1.08$  gradient after incubation at 4°C with AET-treated SRBC. Red cells were removed by hypotonic shock. E<sup>+</sup> cells ( $1 \times 10^5$ ) were cultured in round-bottomed 96-well microtitre plates with various amounts of either fresh E<sup>+</sup> or F<sub>7</sub> T (after 65 days of culture) cells in 200  $\mu$ l of RPMI-1640 (Gibco) supplemented with 10% horse serum and glutamine (200 mM), and A/X31 ( $1 \mu$ g ml<sup>-1</sup>; a) or HKB ( $0.2 \mu$ g ml<sup>-1</sup>; b). Cultures performed in triplicate were maintained at 37°C for 6 days, then washed and recultured in 0.1 ml RPMI medium containing 5% FCS and glutamine. Supernatants were collected after 24 h and assayed for anti-A/X31 or anti-HKB antibodies by a solid-phase enzyme immunoassay. Antibody production was determined by reading the absorbance at 405 nm of the transformed substrate of alkaline phosphatase; actual amounts of antibody were calculated by referring to a standard curve<sup>20</sup>. Results are expressed as a percentage of the optimal response induced by  $10^5$  E<sup>+</sup> cells. For each experimental point, the s.d. was <25% of the mean value. The amount of antibody detected in these experiments ranged from 45.4 to 114 ng ml<sup>-1</sup> (100% A). Backgrounds (E<sup>+</sup> alone) ranged from 4 to 11% of maximal values.

induces B cells to respond, if their appropriate antigen is present.) However, high concentrations of F<sub>7</sub> cells or HF from the supernatant of F<sub>7</sub> (S<sub>7</sub>) sometimes gave small but significant nonspecific help, as shown in Figs 1, 2.

Most reports of antigen-specific HFs indicate that they are not genetically restricted in their action (for review see ref. 8) but some HFs do seem restricted<sup>9,10</sup>. This was investigated using E<sup>+</sup> cells (a mixture of B cells and monocytes) which were either autologous, HLA-DR-semi-compatible or incompatible with the F<sub>7</sub> cells. HF derived from two cell lines (H<sub>2</sub> and F<sub>7</sub>) showed a genetically restricted helper activity stimulating only B cells of certain donors. As shown in Table 2 for F<sub>7</sub> cells and helper factor (S<sub>7</sub>), autologous and both parental B cells were helped, whereas

**Table 2** Restriction of the help effected by F<sub>7</sub> cells and supernatants

| E <sup>+</sup><br>(DR antigen shared<br>with F <sub>7</sub> ) | Source of help (%)                   |                                |                |                |
|---------------------------------------------------------------|--------------------------------------|--------------------------------|----------------|----------------|
|                                                               | None<br>(E <sup>+</sup> cells alone) | E <sup>+</sup><br>(autologous) | F <sub>7</sub> | S <sub>7</sub> |
| 4, 6 (autologous)                                             | 6                                    | 100                            | 93             | 62             |
| 4, 6 (unrelated)                                              | 5                                    | 100                            | 82             | 104            |
| 4 (parent (3, 4))                                             | 7                                    | 100                            | 41             | 47             |
| 4 (1/4)                                                       | 8                                    | 100                            | 96             | 91             |
| 4 (3/4)                                                       | 5                                    | 100                            | 79             | 92             |
| 4 (3/4)                                                       | 14                                   | 100                            | 99             | 90             |
| 4 (4/10)                                                      | 7                                    | 100                            | 85             | 68             |
| 4 (4/5)                                                       | 10                                   | 100                            | 74             | 73             |
| 4 (4/7)                                                       | 8                                    | 100                            | 93             | 90             |
| 4 (4/7)                                                       | 7                                    | 100                            | 77             | 68             |
| 6 (parent) (6/7)                                              | 6                                    | 100                            | 48             | 42             |
| 6 (6/7)                                                       | 5                                    | 100                            | 7              | 7              |
| 6 (1/6)                                                       | 5                                    | 100                            | 10             | 12             |
| 6 (2/6)                                                       | 7                                    | 100                            | 12             | 12             |
| 6 (3/6)                                                       | 12                                   | 100                            | 74             | 94             |
| None (3/7)                                                    | 9                                    | 100                            | 11             | 7              |
| None (2/7)                                                    | 5                                    | 100                            | 7              | 10             |
| None (3/7)                                                    | 11                                   | 100                            | 10             | 14             |
| None (2/-)                                                    | 8                                    | 100                            | 15             | 9              |
| None (2/7)                                                    | 4                                    | 100                            | 88             | 62             |
| None (2/-)                                                    | 10                                   | 100                            | 12             | 7              |
| None (2/7)                                                    | 9                                    | 100                            | 8              | 11             |
| None (2/10)                                                   | 9                                    | 100                            | 8              | 11             |
| None (1/-)                                                    | 6                                    | 100                            | 8              | 5              |

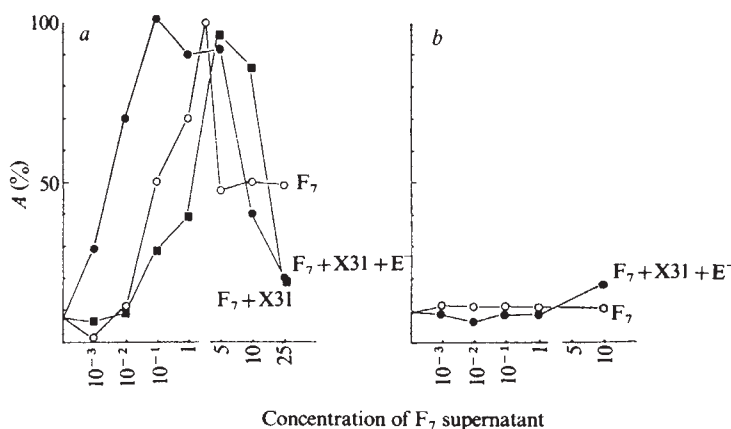
Either F<sub>7</sub> T cells or supernatants (prepared in the presence of irradiated E<sup>+</sup> cells and A/X31) were assayed as described previously<sup>20</sup> on E<sup>+</sup> cells sharing either two, one or no HLA-DR antigens with F<sub>7</sub> T cells. The data are expressed as a percentage of the optimal response provided by E<sup>+</sup> cells autologous to the relevant E<sup>+</sup> cells. For each donor the maximal help provided by the optimal number of F<sub>7</sub> T cells and optimal concentration of supernatant (S<sub>7</sub>) is given. Each individual was HLA typed on two occasions with identical results. All donors were tested at least twice over a wide range of cell and factor concentrations. The 2/7 responder has been tested 4 times with F<sub>7</sub> cells and twice with S<sub>7</sub>.

most HLA-DR-incompatible donor B cells (8 out of 9) were not. All HLA-DR4 B cells tested (10) were strongly helped by F<sub>7</sub> cells and S<sub>7</sub> HF, suggesting either identity or a strong linkage disequilibrium between the restriction element and DR4, as only one of the DRW6-positive B cells except for the parental DR6/7 cells were helped. The latter result could be explained by the wide heterogeneity of the DRW6 group<sup>11</sup>. However, B cells from one of nine DR-incompatible donors were consistently helped by F<sub>7</sub> to produce anti-A/X31 antibodies.

This indicates a close association, but not identity, between the locus coding for T-B restriction and the HLA-DR locus. The nature of the component expressing the genetic restriction is unknown, but as HLA-DR antigens seem to have homology with I-E/C products<sup>12</sup>, the restricting element detected in our system may be the equivalent of the mouse I-A region, which is known to be intimately involved in T-helper cell and factor function<sup>8,13</sup>. The p29, 34 molecule recently described by Nadler *et al.*<sup>14</sup> would be a likely candidate.

The results obtained with long-term cultured T-cell lines specific for influenza strain A/X31 indicate clearly that, just as in the mouse, antigen-specific helper cells can be propagated long term (for over 4 months) using TCGF. A report of human, non-antigen-specific T cells has been published elsewhere<sup>15</sup>. The type of helper cell cultured varies: Schreier and Tees<sup>1,7</sup> and Watson<sup>2</sup> have reported erythrocyte-specific T cells which, while responding to antigen specifically, did not help in a specific manner. In contrast, Eshar *et al.*<sup>3</sup> described a helper line specific for poly(L-tyrosine-L-glutamic acid)-poly(D,L-alanine)-poly(L-lysine) (TGAL) and P. Erb (personal communication) has derived a keyhole-limpet haemocyanin-specific helper line which produces antigen-specific HF. It seems likely that these contrasting results, which closely parallel discrepancies concerning mechanisms of T-cell help, reflect different subpopulations of helper cells.

Our results have been obtained with a long-term cell line, and not with clones. Many attempts to clone these cells at low numbers, in the presence of feeder cells, have failed. It is



**Fig. 2** Helper activity of  $F_7$  supernatants. Long term-cultured T cells ( $F_7$ ) after 80 days of culture ( $1 \times 10^6 \text{ ml}^{-1}$ ) were incubated alone (○) or with optimal dose of antigen A/X31 (■) or with antigen plus irradiated ( $2,500 \text{ rad}$ )  $E^-$  cells ( $1 \times 10^6 \text{ ml}^{-1}$ ; ●) for 18 h in TCGF-free medium containing 10% horse serum. The supernatants were collected by centrifugation, filtered and stored at  $-20^\circ \text{C}$ . They were assayed at various concentrations (expressed as a percentage) for their ability to help the production *in vitro* of anti-A/X31 (a) or anti-HKB (b) by autologous  $E^-$  cells, as described in Fig. 1 legend. Results are expressed as a percentage of the optimal response induced by  $10^5 E^-$  cells.

conceivable that these helper cells will not grow in the absence of another 'amplifier cell', also of the  $3a^+$  phenotype.

The genetically restricted nature of the specific helper factor obtained here is intriguing, and is comparable with other results in the mouse<sup>10</sup> and with the specific nature of the help for anti-tetanus antibody production in humans<sup>16</sup>, but is in contrast to many others<sup>8,13</sup>. In view of the genetic restriction repeatedly observed in T-B collaboration, we feel that further analysis of these cell lines should elucidate the nature of T-cell receptors and their factors, and the mechanisms of T-B collaboration.

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- Schreier, M. H., Iscove, N. N., Tees, R., Aarden, L. & von Boehmer, H. *Immun. Rev.* **51**, 315-336 (1980).
- Watson, J. J. *exp. Med.* **150**, 1510-1519 (1979).
- Eshar, Z. *et al. Nature* **286**, 270-272 (1980).
- Kantor, F. & Feldmann, M. *Clin. exp. Immun.* **36**, 71-77 (1979).
- Callard, R. E. *Nature* **282**, 734-736 (1979).
- Abramson, C. S., Kersey, J. H. & Le Bien, T. J. *Immun.* **126**, 83-88 (1981).
- Tees, R. & Schreier, M. H. *Nature* **283**, 780-781 (1980).
- Feldmann, M. & Kontiainen, S. *Lymphokines* **2**, 87-123 (1981).
- Shiozawa, C., Singh, B., Robinson, S. & Diener, E. J. *Immun.* **118**, 2199-2206 (1977).
- Lonai, P., Puri, J. & Hammerling, G. J. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Doran, T. J., Honeyman, M., Zwing, W., Edwards, C. M. & Basher, H. W. *8th Int. Workshop on Histocompatibility*, 808-809 (ed. Terasaki, P. I.) (UCLA Tissue Typing Laboratory, Los Angeles, 1980).
- Bodmer, W. *Tissue Antigens* **17**, 9-20 (1981).
- Munro, A. J. & Taussig, M. S. *Nature* **256**, 103-106 (1975).
- Nadler, L. M. *et al. Nature* **290**, 591-593 (1981).
- Friedmann, S. M., Hunter, S. B., Thomas, Y. & Chess, L. J. *Immun.* **125**, 2202-2207 (1980).
- Geha, R. S. *Immun. Rev.* **45**, 275-305 (1979).
- Kurnick, J. T. *et al. Scand J. Immun.* **11**, 131-136 (1980).
- Beverly, P. C. L. & Callard, R. E. *Eur. J. Immun.* **11**, 329-334 (1981).
- Brodsky, F. M., Parham, P., Barnstable, C. J., Crumpton, M. J. & Bodmer, W. F. *Immun. Rev.* **47**, 3 (1979).
- Zanders, E. D., Smith, C. M. & Callard, R. E. *J. immun. Meth.* (in the press).

## Antibody directed at a surface structure inhibits cytolytic but not suppressor function of human T lymphocytes

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Experiments using conventional antisera<sup>1,2</sup> and monoclonal antibodies<sup>3-5</sup> have shown that there are two functionally distinct human T-cell subsets which express unique cell-surface glycoproteins<sup>6-9</sup>. The inducer population expresses a 62,000-molecular weight ( $M_r$ ) antigen termed T4 (ref. 6) whereas the cytotoxic/suppressor population lacks T4 but expresses T5 antigen. This antigen is a 76,000- $M_r$  molecule in nonreducing conditions and a 33,000- $M_r$  glycoprotein occasionally appearing as a doublet in reducing conditions. As the functions of subset-specific T-cell surface glycoproteins have not been elucidated, we have now examined the effect on T-cell function of three monoclonal antibodies reactive with the cytotoxic/suppressor subset; anti-T8<sub>A</sub> antibody markedly inhibited cell-mediated lympholysis (CML) and anti-T8 partially affected CML, whereas anti-T5 had no effect. In contrast, there was no effect on suppressor cell function. Immunoprecipitation studies and competitive binding experiments indicated that anti-T8 and anti-T8<sub>A</sub>, like anti-T5, defined a 33,000- $M_r$  antigen. Taken together, our results suggest that of a possible family of 33,000- $M_r$  glycoproteins on the human cytotoxic/suppressor T-lymphocyte subset, that which is reactive with anti-T8<sub>A</sub> has some effect on the cytolytic mechanism.

Effector function for CML resides in the subset of T lymphocytes recognized by the antibodies anti-T5 and anti-T8 (refs 3, 4, 9), which are monoclonal hybridoma products of IgG1 and IgG2a isotypes, respectively<sup>4,5</sup>. Elimination of the subsets of cells reactive with these antibodies by fluorescence-activated cell sorting (FACS) or, in the case of anti-T8, complement-mediated lysis, was found to abrogate the cytotoxic effector function of the unfractionated T-cell population<sup>9</sup>. Here, an additional monoclonal antibody, anti-T8<sub>A</sub>, was developed by standard hybridoma techniques after immunization of a BALB/cJ mouse with human thymocytes as described previously<sup>10</sup>. Hybridoma cultures containing antibody reactive with  $E^+$  or thymocyte populations or both were selected, cloned and recloned by limiting dilution methods in the presence of feeder cells. Malignant ascites were then developed and used for analysis. Anti-T8<sub>A</sub> represents an ascites from one hybridoma clone (SFCl 21Thy). The pattern of reactivity of the anti-T8<sub>A</sub> was identical to that described for anti-T8 (ref. 5) as it defined ~80% of thymocyte and 30-35% of peripheral T cells and was unreactive with B cells, null cells and macrophages by indirect immunofluorescence.

To determine whether the antigens defined by these antibodies were themselves important for the cytotoxic response of T lymphocytes, the following procedure was used. T cells were sensitized to alloantigens in mixed lymphocyte culture (MLC) for 6 days. Subsequently, the cytotoxic cells (CTL) were collected, incubated with a 1:1,000 dilution of a given monoclonal antibody or control and their ability to lyse <sup>51</sup>Cr-labelled target cells was investigated. In the absence of monoclonal antibody (control), specific lysis in primary CML was ~28-29% at either

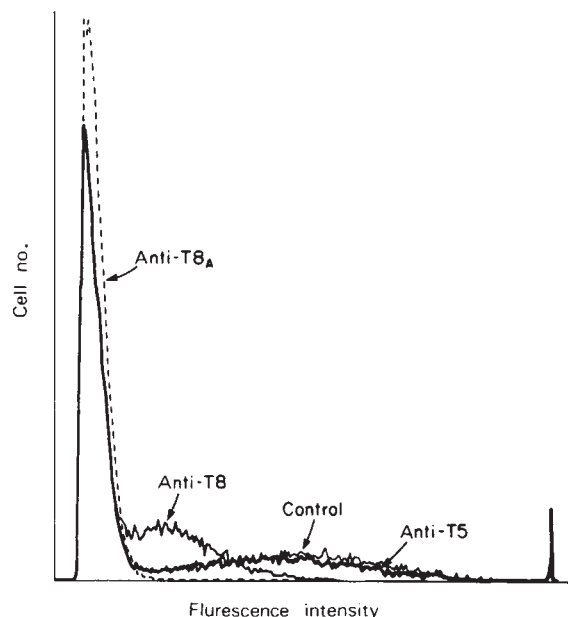
**Table 1** Effect on % specific lysis of preincubation of CTL with monoclonal antibodies

| Monoclonal antibody  | % Specific lysis at an effector/target ratio of: |      |       |
|----------------------|--------------------------------------------------|------|-------|
|                      | 40:1                                             | 20:1 | 10:1  |
| Control              | 28±2                                             | 29±1 | 21±1  |
| Anti-T5              | 30±2                                             | 28±2 | 22±2  |
| Anti-T8              | 16±2                                             | 14±2 | 15±1  |
| Anti-T8 <sub>A</sub> | 2±1                                              | 2±1  | 0.7±1 |

Peripheral T cells were activated in one-way MLC with mitomycin C-treated allogeneic human mononuclear cells as previously described<sup>10</sup>. Subsequently, alloantigen-activated killer cells were collected and tested in a <sup>51</sup>Cr microcytotoxicity assay for 4 h (ref. 3). Specificity of CML was tested by using an additional allogeneic target population unrelated to the mononuclear population used in the initial sensitization phase of MLC. The level of nonspecific killing was always ≤10% of specific killing. To determine whether anti-T5, anti-T8 or anti-T8<sub>A</sub> antibody inhibited CML in the absence of complement, some killer cells were incubated with a 1:1,000 dilution of monoclonal antibody or control ascites at 20 °C for 30 min before addition of the relevant target cells. The control ascites was obtained from BALB/cJ mice immunized with a non-producing hybridoma clone<sup>10</sup>.

a 40:1 or 20:1 effector/target cell ratio but was reduced to 21% at a 10:1 effector/target ratio (see Table 1). Preincubation of cytotoxic cells with anti-T5 did not affect the magnitude of this response, whereas preincubation of 6-day MLC-stimulated cells with anti-T8 resulted in a diminished percentage specific lysis such that, even at a 40:1 effector/target cell ratio, only 16% of target cells were killed. A similar percentage lysis was obtained if the effectors were preincubated with anti-T8 and used at an effector/target ratio of 20:1 or 10:1. In contrast, the anti-T8<sub>A</sub> monoclonal antibody almost completely inhibited killing. These findings indicate that anti-T5 has no inhibitory effect on the effector phase of CML whereas the anti-T8 and anti-T8<sub>A</sub> monoclonal antibodies have a moderate to marked effect, respectively. These effects were seen at dilutions of monoclonal antibody between 10<sup>-2</sup> and 10<sup>-4</sup>. As anti-T5 and anti-T8<sub>A</sub> monoclonal antibodies are of the IgG1 subclass<sup>5</sup>, the results cannot be explained by differences in immunoglobulin isotype.

Anti-T8<sub>A</sub> had no effect on CTL effector function when added at the initiation of MLC (data not shown). In contrast, anti-T3 regularly blocked CTL generation in MLC and generally also blocked at the effector phase<sup>11-15</sup>. The observation that anti-T3, which defines a 19,000-M<sub>r</sub> glycoprotein, and anti-T8<sub>A</sub>, which defines a 33,000-M<sub>r</sub> protein, both inhibit cytolysis suggests that multiple cell surface antigens are important in mediation of CML.



**Fig. 1** Competitive blocking studies with monoclonal antibodies to cytotoxic/suppressor cells. The relationships between anti-T5, anti-T8 and anti-T8<sub>A</sub> antibodies were examined in competitive binding studies by direct immunofluorescence using FACS<sup>10</sup>. T cells (1×10<sup>6</sup>) were treated with 0.15 ml of a 1:100 dilution of either control ascites, anti-T5, anti-T8 or anti-T8<sub>A</sub>, incubated at 4 °C for 30 min and washed twice. The control ascites was obtained from BALB/cJ mice (see Table 1). The cells were reacted with 0.15 ml of a 1:50 dilution of directly fluoresceinated anti-T8<sub>A</sub> at 4 °C for 30 min, centrifuged and washed three times. Subsequently, 40,000 cells were analysed on the FACS I (Becton Dickinson) and the intensity of fluorescence per cell recorded on a pulse height analyser.

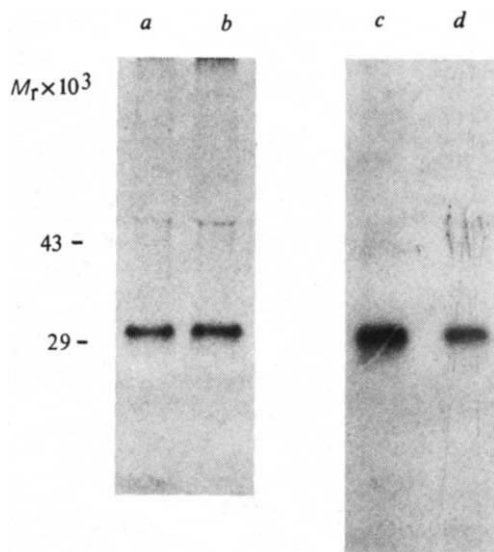
As T5(T8<sup>+</sup>) T cells have been observed<sup>8</sup> to suppress pokeweed mitogen (PWM) induced immunoglobulin synthesis, we examined whether antibodies to the T5, T8 or T8<sub>A</sub> antigen could affect this suppressor function. B cells and T4<sup>+</sup> inducer T cells were cultured with various numbers of T8<sup>+</sup> T cells in the presence of the particular monoclonal antibody and stimulated with PWM. Immunoglobulin production was measured by radioimmunoassay after 7 days of culture. As expected (Table 2), T4<sup>+</sup> T cells induced B-cell immunoglobulin production whereas T8<sup>+</sup> T cells did not (11,000 as opposed to <200

**Table 2** Anti-T5, -T8 and -T8<sub>A</sub> antibodies do not inhibit suppressor T-cell function in T-B interactions

| B cells | Cells in PWM culture        |                             | IgG (ng ml <sup>-1</sup> ) secreted over 7 days in the presence or absence of monoclonal antibodies |         |         |                      |
|---------|-----------------------------|-----------------------------|-----------------------------------------------------------------------------------------------------|---------|---------|----------------------|
|         | T4 <sup>+</sup> cells added | T8 <sup>+</sup> cells added | Control                                                                                             | Anti-T5 | Anti-T8 | Anti-T8 <sub>A</sub> |
| +       | 0                           | 2×10 <sup>4</sup>           | ≤200                                                                                                | ≤200    | ≤200    | ≤200                 |
| +       | 2×10 <sup>4</sup>           | 0                           | 11,800                                                                                              | 11,400  | 11,600  | 11,400               |
| +       | 2×10 <sup>4</sup>           | 1×10 <sup>4</sup>           | 8,500                                                                                               | 8,600   | 8,400   | 8,300                |
| +       | 2×10 <sup>4</sup>           | 5×10 <sup>4</sup>           | 4,000                                                                                               | 4,100   | 4,300   | 3,900                |
| +       | 2×10 <sup>4</sup>           | 10×10 <sup>4</sup>          | 300                                                                                                 | 400     | ≤200    | ≤200                 |
| -       | 2×10 <sup>4</sup>           | 0                           | ≤200                                                                                                | ≤200    | ≤200    | ≤200                 |

Unfractionated mononuclear cells were separated into T- and B-enriched populations as previously described<sup>7</sup>. The B-cell population was <1% E-rosette positive. The T4<sup>+</sup> and T8<sup>+</sup> T-cell subsets were obtained by complement-dependent lysis of unfractionated T cells with anti-T8 and anti-T4, respectively, and were ≥93% pure on re-analysis<sup>9</sup>. To 5×10<sup>4</sup> B cells or final medium (RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (Microbiological Associates), 200 mM L-glutamine, 25 mM HEPES buffer (Microbiological Associates), 0.5% sodium bicarbonate and 1% penicillin-streptomycin) were added varying numbers of T4<sup>+</sup> and T8<sup>+</sup> T cells in a total volume of 0.1 ml with 0.1 ml PWM (Gibco) diluted 1:50 in final medium containing control ascites (1:100), anti-T5 (1:100), anti-T8 (1:100) or anti-T8<sub>A</sub> (1:100). After 7 days in culture at 37 °C in a humid atmosphere with 5% CO<sub>2</sub>, culture supernatants were collected and IgG secretion during the 7-day period determined by solid phase radioimmunoassay<sup>8</sup>.





**Fig. 2** Characterization and isolation of the T5, T8 and T8<sub>A</sub> antigens. The external proteins of the human T-cell line HPB-ALL were labelled with <sup>125</sup>I using IODO-GEN (Pierce). Labelled cells washed three times with phosphate-buffered saline containing soybean trypsin inhibitor (Sigma), 2 mM phenylmethylketonesulphate (PMKS) and 1 mM iodoacetamide (Pierce) were extracted with 1% Nonidet-P40 (NP40; Partide Data Laboratories, Elmhurst, Illinois) in 0.01 M Tris-HCl pH 8.0, 2 mM PMKS, trypsin inhibitor and 1 mM iodoacetamide at 0°C for 30 min. Nuclear debris was removed in an Eppendorf centrifuge (15 min at 12,000g) and the supernatant cleared in an Airfuge (30 min at 100,000g; Beckman). Immunoprecipitations were carried out by a technique which uses preformed complexes prepared from rabbit anti-mouse IgG serum and the monoclonal reagent anti-T5, anti-T8 or anti-T8<sub>A</sub> as described elsewhere<sup>6</sup>. Immunoprecipitates were washed with 0.01 M Tris-HCl pH 8.0, 0.59% deoxycholate, 2 mM PMKS and 0.01 M Tris-HCl pH 8.0, 0.2% NP40. The surface antigens immunoprecipitated with the anti-T5, anti-T8 and anti-T8<sub>A</sub> monoclonal antibodies were analysed by SDS-polyacrylamide gel electrophoresis on 5–15% gradient gels. a, Anti-T8; b, anti-T8<sub>A</sub>; c, anti-T8; d, anti-T5.

ng ml<sup>-1</sup>, respectively, secreted over 7 days). Furthermore, when increasing numbers of T8<sup>+</sup> T cells were added to a mixture of 2 × 10<sup>4</sup> T4<sup>+</sup> T cells and 5 × 10<sup>4</sup> B cells, immunoglobulin production was markedly suppressed. When 5 × 10<sup>4</sup> T8<sup>+</sup> T cells were added to the T4<sup>+</sup>-B cell mixture, immunoglobulin production decreased by >50% to 4,000 ng. IgG secretion was virtually abolished (300 ng ml<sup>-1</sup>) when 1 × 10<sup>5</sup> T8<sup>+</sup> T cells were added to the T4<sup>+</sup>-B cell population. More importantly, preincubation of the mixture of T and B cells with anti-T5, anti-T8 or anti-T8<sub>A</sub> had no effect on the suppression caused by the T8<sup>+</sup> T-cell subset. Thus, it seems that the inhibitory effect of anti-T8<sub>A</sub> is selective for cytotoxic effector, rather than suppressor, function. However, the two assays are not comparable as the former occurs over 4 h and the latter over 7 days.

To determine whether these monoclonal antibodies defined related or distinct sets of antigens, the ability of one monoclonal antibody to inhibit binding of a second was determined by direct immunofluorescence. As shown in Fig. 1, ~30% of peripheral T cells were reactive with fluorescein isothiocyanate-conjugated (FITC)-labelled anti-T8<sub>A</sub> in a FACS. Preincubation of T cells with unlabelled anti-T8<sub>A</sub> before addition of FITC-anti-T8<sub>A</sub> totally inhibited the binding of the latter. In contrast, preincubation with unlabelled anti-T5 was not inhibitory. Preincubation of T cells with anti-T8 antibody showed a partial inhibition of FITC-anti-T8<sub>A</sub> binding. Similarly, preincubation of T cells with anti-T8<sub>A</sub> inhibited binding of FITC-anti-T8. These results suggest that the determinant defined by anti-T5 is

distinct from that defined by anti-T8<sub>A</sub> and that anti-T8 and anti-T8<sub>A</sub> seem to react with a related structure.

The molecular nature of these antigens was then examined in a second series of experiments. Solubilized membrane preparations were obtained from the externally labelled human T-cell line HPB-ALL and the antigens defined by anti-T5, anti-T8 and anti-T8<sub>A</sub> antibodies precipitated and electrophoresed on SDS-polyacrylamide gels. As previously reported<sup>6</sup>, the antigen precipitated by anti-T5 antibody was a 33,000-*M<sub>r</sub>* protein in reducing conditions (Fig. 2) and a 76,000 *M<sub>r</sub>* protein in non-reducing conditions (data not shown). The anti-T8 and anti-T8<sub>A</sub> antibodies precipitated molecules of the same molecular weight (Fig. 2). Although these antigens appear similar and, in sequential precipitation studies anti-T8<sub>A</sub> largely removed both the T8<sub>A</sub> and T8 antigens, anti-T5 did not deplete T8 antigen (data not shown). These results suggest that the T5 and T8 antigens are not identical. A similar conclusion was reached after analysis of T-ALL tumour cells using anti T5 and anti-T8 in which some cell were reactive with anti-T8 but unreactive with anti-T5 (ref. 5).

Both the biochemical data and binding studies are consistent with the view that the T8 and T8<sub>A</sub> epitopes are located close together on a single molecule. However, proof of this conclusion must await peptide mapping and two-dimensional gel analysis. Nevertheless, the inhibition of CTL function implies that the epitope defined by anti-T8<sub>A</sub> may be critical for cytolytic function but not for suppressor function. Thus, these antigens seem to be homologous to Lyt2, which has been shown to inhibit murine CTL to varying degrees<sup>16,17</sup>. A recent report<sup>18</sup> suggests that another antibody defining the human cytotoxic cell population, termed anti-Leu2a, blocks CML. It is possible that this antibody may be directed against the same 33,000-*M<sub>r</sub>* protein.

The ability of anti-T8<sub>A</sub> to inhibit CML at the effector phase may involve blocking of structures near or associated with the antigen receptor of the T cell. Alternatively, Lyt2 or T8<sub>A</sub> may serve as molecules permitting attachment for conjugate formation between killer and target cells without themselves being antigen-specific receptors<sup>19</sup>. Further experiments using purified and reconstituted T8<sub>A</sub> antigens will help to resolve these issues.

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- Evans, R. L., Lazarus, H., Penta, A. C. & Schlossman, S. F. *J. Immun.* **120**, 1423–1428 (1978).
- Reinherz, E. L. & Schlossman, S. F. *J. Immun.* **122**, 1335–1341 (1979).
- Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4061–4065 (1979).
- Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. *J. Immun.* **124**, 1301–1307 (1980).
- Reinherz, E. L., Kung, P. C., Goldstein, G., Levey, R. & Schlossman, S. F. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1588–1592 (1980).
- Terhorst, C., van Agthoven, A., Reinherz, E. L. & Schlossman, S. F. *Science* **209**, 520–521 (1980).
- Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. *J. Immun.* **123**, 2894–2896 (1979).
- Reinherz, E. L., Morimoto, C., Penta, A. C. & Schlossman, S. F. *Eur. J. Immun.* **10**, 570–572 (1980).
- Reinherz, E. L., Hussey, R. E. & Schlossman, S. F. *Immunogenetics* **11**, 421–426 (1980).
- Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. *J. Immun.* **123**, 1312–1317 (1979).
- Reinherz, E. L., Kung, P. C., Pesando, J. M., Ritz, J., Goldstein, G. & Schlossman, S. F. *J. exp. Med.* **150**, 1472–1482 (1979).
- Kung, P. C., Goldstein, G., Reinherz, E. L. & Schlossman, S. F. *Science* **206**, 347–349 (1979).
- van Agthoven, A., Terhorst, C., Reinherz, E. L. & Schlossman, S. F. *Eur. J. Immun.* **11**, 18–21 (1981).
- Reinherz, E. L., Hussey, R. E. & Schlossman, S. F. *Eur. J. Immun.* **10**, 758–762 (1980).
- Chang, T. W., Kung, P. C., Gingras, S. P. & Goldstein, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1805–1808 (1981).
- Nakoyama, E., Shiku, H., Stockert, E., Oettgen, H. F. & Old, L. J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1977–1981 (1979).
- Shinohara, N. & Sachs, D. H. *J. exp. Med.* **150**, 432–444 (1979).
- Evans, R. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 544–548 (1981).
- Samiento, M., Glasebrook, A. L. & Fitch, F. W. *J. Immun.* **125**, 2665–2672 (1980).



# Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumour cells

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The development of the monoclonal antibody technique<sup>1</sup> has led to renewed interest in whether the growth of tumour cells may be specifically inhibited by antibodies or antibody-toxin conjugates directed against antigenic determinants selectively expressed on tumour cells<sup>2-10</sup>. We have recently obtained monoclonal antibodies against the transferrin receptor of human cells<sup>11,12</sup>, which is thought to have an essential role in transport of Fe across the cell membrane and which is selectively expressed on proliferating cells *in vivo* and *in vitro*<sup>13-17</sup>. In some cases, transferrin receptors can be used as a marker to distinguish between tumour cells and normal tissue<sup>11,15-17</sup>. Here we show that human tumour cells are specifically killed *in vitro* by anti-transferrin receptor antibody covalently coupled to ricin or diphtheria toxic subunits. In experiments designed to test the effectiveness of these antibody-toxin conjugates *in vivo*, we found that anti-transferrin receptor antibody alone inhibits the growth of human melanoma cells in nude mice.

The mouse monoclonal IgG1  $\kappa$  antibody against the human transferrin receptor, designated B3/25, has been described previously<sup>11,12</sup>. The antibody was purified from ascitic fluid of tumour-bearing mice by precipitating with ammonium sulphate and fractionation on DEAE-cellulose. Ricin A toxic subunit was prepared from affinity-purified ricin toxin by reduction with 2-mercaptoethanol and fractionation on DEAE- and CM-cellulose<sup>18</sup>. Diphtheria fragment A was prepared from diphtheria toxin (Connaught Laboratories) by tryptic cleavage<sup>19</sup> followed by reduction with 2-mercaptoethanol and chromatography on Sephacryl S-200. Antibody-toxin conjugates were synthesized using *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pharmacia)<sup>20</sup>. A sixfold molar excess of SPDP was used to introduce an average of three 2-pyridyldisulphide groups into the anti-receptor antibody. The modified antibody was then mixed with a threefold molar excess of freshly reduced ricin A subunit or diphtheria fragment A and incubated at 4°C for 36 h. The antibody conjugate was purified from unbound toxin subunits by chromatography on Sephacryl S-200. On average, 1-2 toxin subunits were covalently bound to each antibody molecule.

The effect of anti-transferrin receptor antibody-ricin A conjugate on protein synthesis in a human T leukaemic cell line, CCRF-CEM<sup>21</sup>, is shown in Fig. 1. It can be seen that the antibody-ricin A conjugates inhibit protein synthesis almost as effectively as the intact toxin. The residual protein synthesis in cells treated with high concentrations of the conjugate reflects the fact that the conjugate seems to act less rapidly than the intact toxin and is not observed if cells are exposed to the conjugate for 48 h. It is possible that cells differ in susceptibility to the antibody-ricin A conjugate depending on their position in the cell cycle. Antibody or ricin A subunit added alone or as a mixture, inhibited protein synthesis only at concentrations 200-1,000-fold higher than the antibody-ricin A conjugate. The specificity of the antibody-ricin A conjugates is further indicated by the fact that protein synthesis in a mouse lymphoma cell line, BW5147, was not inhibited by the anti-human transferrin receptor antibody-ricin A conjugate even though the cells were as sensitive as CCRF-CEM cells to the intact ricin toxin (Table 1). Specific inhibition of protein synthesis in CCRF-

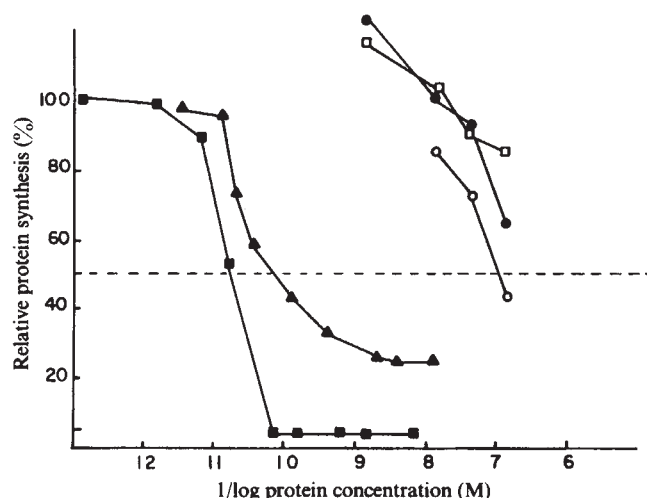


Fig. 1 Protein synthesis in a human leukaemic cell line is inhibited by anti-transferrin receptor-ricin A conjugate. CCRF-CEM cells ( $1 \times 10^6$ ) were cultured for 20 h in 2.0 ml RPMI 1640 medium supplemented with 10% horse serum and various concentrations of ricin toxin (■), anti-transferrin receptor plus ricin A conjugate (▲), ricin A subunit (●), anti-transferrin receptor antibody (□), or anti-transferrin receptor antibody plus ricin A subunit (○). Then 10  $\mu$ Ci of  $^3$ H-leucine (42 Ci mmol<sup>-1</sup>, NEN) were added to each dish and the cells were incubated for 2 h. Cells from duplicate cultures for each treatment were collected and incorporation of radioactivity into trichloroacetic acid-insoluble material was determined. Protein synthesis in treated cells was expressed as a percentage of  $^3$ H-leucine incorporation into untreated control cells. Note that although ricin A was diluted in this experiment from a stock solution at 250  $\mu$ g ml<sup>-1</sup> in 0.1% mercaptoethanol, in earlier experiments dialysis was carried out to remove the reducing agents without increasing the toxicity of the free ricin A chain.

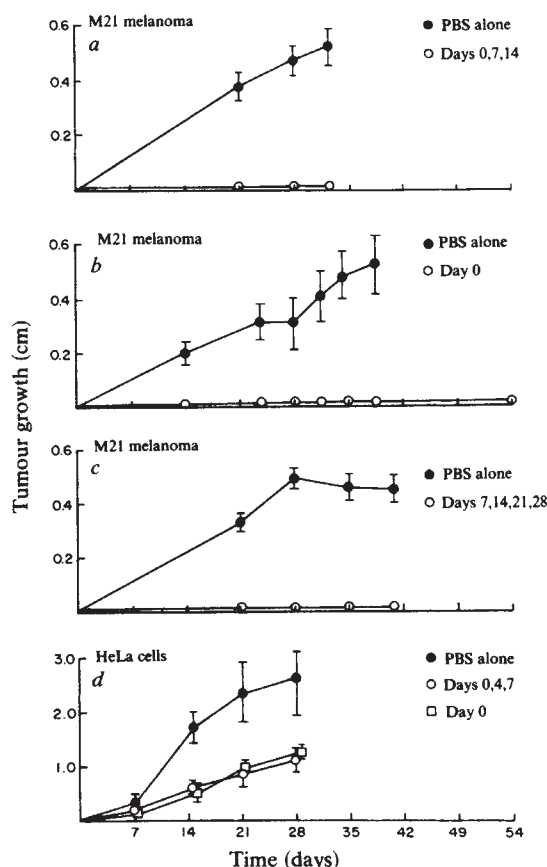
CEM cells was also obtained with an anti-receptor antibody-diphtheria fragment A conjugate. Protein synthesis in HeLa cells and cells of a human melanoma cell line (M21)<sup>22</sup>, was inhibited by anti-receptor antibody-ricin A, although M21 cells seemed less sensitive than CCRF-CEM cells.

As a further test of the ability of the anti-receptor antibody-ricin A conjugate to kill tumour cells *in vitro*, and to assess the frequency with which cells might escape killing, we performed cloning assays. CCRF-CEM cells were plated out in twofold dilutions at concentrations ranging from  $5-1 \times 10^6$  cells per ml in 0.2 ml tissue culture medium in microtitre wells. No growth was observed even in wells containing  $2 \times 10^5$  cells in the presence of anti-receptor-ricin A antibody (10 nM), whereas a cloning efficiency of ~30% was observed in the absence of the con-

Table 1 Inhibition of protein synthesis in human tumour cell lines by anti-transferrin receptor-toxic subunit conjugates

| Cell line                  | Antibody-diphtheria A | IC <sub>50</sub>               |                   |
|----------------------------|-----------------------|--------------------------------|-------------------|
|                            |                       | Antibody-ricin A               | Ricin toxin alone |
| CCRF-CEM human T leukaemia | 1.5                   | 0.06, 0.04, 0.09, 0.03*, 0.06* | 0.03              |
| M21 human melanoma         | —                     | 3.5                            | —                 |
| HeLa                       | —                     | 0.25                           | —                 |
| BW5147 mouse lymphoma      | > 11                  | > 13                           | 0.04              |

The concentration (nM) required for 50% inhibition of protein synthesis (IC<sub>50</sub>) was calculated from results obtained in experiments of the type shown in Fig. 1. The same preparation of ricin A-antibody conjugate was used for all experiments, except for the two IC<sub>50</sub> values for CCRF-CEM cells marked with an asterisk: a separate preparation of conjugate was used in these two experiments.



**Fig. 2** Inhibition of tumour growth in nude mice by anti-transferrin receptor monoclonal antibody. Groups of BALB/c (*nu/nu*) mice (4 mice per group, females 4–5 weeks old) were given subcutaneous inoculations of  $2 \times 10^7$  M21 melanoma or HeLa cells. Anti-transferrin receptor monoclonal antibody or antibody-ricin A conjugate was then given i.v. in 0.2 ml phosphate-buffered saline (PBS) either immediately (day 0) or at the times indicated (open symbols). In *a*, 50 µg of ricin A-antibody conjugate was injected each time. For experiments *b–d*, 880 µg of unmodified antibody was given on each occasion. Control mice were injected with PBS alone (solid symbols). Tumour growth was estimated by measuring two perpendicular diameters of the tumours using calipers; results are expressed as geometric mean  $\pm$  1 s.e.

jugate. In similar experiments, no survivors were obtained when  $2 \times 10^5$  HeLa cells were treated with the same concentration of the antibody conjugate. In contrast, no effect was observed on the cloning efficiency of BW5147 mouse lymphoma cells.

As the antibody-toxic subunit conjugates were effective in killing human tumour cells *in vitro*, we set out to determine whether the antibody-ricin A conjugates were effective in inhibiting the growth of human tumour cells in nude mice. As CCRF-CEM cells do not produce tumours in nude mice, M21 and HeLa cells were used. We found that the antibody-ricin A conjugate (50 µg intravenously (i.v.) on days 0, 7, 14) completely inhibited the growth of M21 cells in nude mice (Fig. 2*a*). However, a single i.v. injection of 880 µg of anti-transferrin receptor antibody alone also inhibited the growth of the melanoma cells at a subcutaneous site (Fig. 2*b*). The same results have been obtained in four similar experiments. In a fifth experiment, tumour formation was delayed but not completely inhibited. As shown in Fig. 2*c*, administration of antibody 7 days after inoculation of tumour cells was also effective in preventing tumour growth. However, the monoclonal anti-transferrin receptor antibody was much less effective in inhibiting growth of HeLa cells in nude mice even when repeated doses of antibody were given (Fig. 2*d*).

In another experiment, groups of mice were given a single i.v. injection at day 0 of either 10 or 50 µg of antibody or antibody-ricin A conjugate. Mice given the higher dose of either

unmodified antibody or conjugate did not develop tumours, whereas tumour growth in those mice receiving lower concentrations of each reagent was not significantly different from that of the controls. Thus, there is no evidence that the conjugate is more effective than unmodified antibody in inhibiting growth of the M21 melanoma cells in nude mice. However, this may reflect instability of the antibody-ricin A conjugate *in vivo*, or differences in the relative clearance of unmodified and conjugated antibody from the circulation, and requires further study. Furthermore, although the antibody-ricin A conjugate inhibits growth of HeLa cells *in vitro*, it is no more effective than antibody alone in preventing the growth of HeLa cell tumours in nude mice.

Recent reports have indicated that specific killing of tumour cells can be achieved *in vitro* with antibody-toxin conjugates<sup>2–5,7</sup>. We have shown here that similar results can be obtained with anti-human transferrin receptor antibodies coupled to ricin A subunit or diphtheria fragment A. More importantly, we have shown that growth of a human melanoma cell line in nude mice can be effectively inhibited by anti-human transferrin receptor antibody alone. In contrast, previous attempts to inhibit growth of murine lymphomas by administration of either anti-Thy-1 antibody<sup>5</sup> or ricin A-anti-Thy-1 antibody conjugates<sup>7</sup> were only partially effective. Whether inhibition of M21 tumour growth is due to immunological mechanisms or to the effect of antibody on the ability of the tumour cells to compete for Fe *in vivo* remains to be determined. Clearly, however, sufficient antibody to inhibit growth can be delivered to the subcutaneous tumour by an i.v. injection. Furthermore, it seems that *in vitro* tests are of limited value in predicting the *in vivo* effects of antibody reagents on tumour growth.

Our preliminary results and the known properties of the transferrin receptor suggest that further studies of the usefulness of anti-receptor antibodies as therapeutic agents would be worthwhile. The transferrin receptor is a major glycoprotein on dividing tumour cells and is not rapidly shed from the cell surface (M. B. Omary and I. S. T., unpublished results). It is probably essential for cell growth, thus it is unlikely that tumour cells would be able to escape killing either by antigenic modulation<sup>23</sup> or by genetic loss of receptors<sup>24</sup>. As it is thought that receptor-bound transferrin is internalized<sup>25,26</sup>, the transferrin receptor might be expected to facilitate entry of bound antibody conjugates into the cell. Furthermore, anti-receptor antibodies may exert inhibitory effects on growth by interfering with Fe transport. Finally, as transferrin receptors are known to be present on mouse tumour cells, suitable animal models can be used to test the effectiveness of anti-receptor monoclonal antibody on *in vivo* growth of transplanted and spontaneous tumours and to examine the potential deleterious effects of antibody on normal dividing cells. A rat monoclonal antibody to the mouse transferrin receptor has recently been obtained (I.S.T., J. Lesley and R. Schulte, unpublished results).

Although the transferrin receptor is obviously not a tumour-specific antigen, there is little evidence that such antigens exist in man, and careful studies have always revealed that putative tumour-specific antigens are expressed on some normal cells<sup>27–29</sup>. Indeed, the transferrin receptor itself may be the putative cell surface glycoprotein associated with malignancy described elsewhere<sup>30,31</sup>. Given this situation, it is probable that absolute specificity of anti-tumour antibody reagents is unlikely to be achieved. However, sufficient specificity may be obtained using monoclonal antibodies against target antigens such as the transferrin receptor, alone or in combination, to be of practical therapeutic importance.

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- Köhler, G. & Milstein, C. *Nature* **256**, 495–497 (1975).
- Gilliland, D. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 4539–4543 (1980).
- Krolick, K. A., Villemez, C., Isakson, P., Uhr, J. W. & Vitetta, E. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5419–5423 (1980).



4. Youle, R. J. & Neville, D. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5483-5486 (1980).
5. Bernstein, I. D., Tam, M. R. & Nowinski, R. C. *Science* **207**, 68-71 (1980).
6. Nadler, L. M. *et al. Cancer Res.* **40**, 3147-3154 (1980).
7. Blythman, H. E. *et al. Nature* **290**, 145-146 (1981).
8. Davies, T. *Nature* **289**, 12-13 (1981).
9. Miller, R. A., Maloney, D. G., McKillop, J. & Levey, R. *Blood* **58**, 79-86 (1981).
10. Ritz, J. *et al. Blood* **58**, 141-151 (1981).
11. Omary, M. B., Trowbridge, I. S. & Minowada, J. M. *Nature* **286**, 888-891 (1980).
12. Trowbridge, I. S. & Omary, M. B. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3039-3043 (1981).
13. Larrick, J. W. & Creswell, P. G. *J. supramolec. Struct.* **11**, 579-586 (1980).
14. Hamilton, T. A., Wada, H. G. & Sussman, H. H. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6406-6410 (1979).
15. Faulk, W. P., Hsi, B.-L. & Stevens, P. J. *Lancet* **ii**, 390-392 (1980).
16. Shindelman, J. E., Ortmeier, A. E. & Sussman, H. H. *Int. J. Cancer* **27**, 329-334 (1981).
17. Sutherland, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 4515-4519 (1981).
18. Olsnes, S. & Pihl, A. *Biochemistry* **12**, 3121-3126 (1973).
19. Kandel, J., Collier, R. J. & Chung, S. W. *J. biol. Chem.* **249**, 2088-2097 (1974).
20. Carlsson, J., Drevin, H. & Axen, R. *Biochem. J.* **173**, 723-737 (1978).
21. Foley, G. E. *et al. Cancer* **18**, 522-529 (1965).
22. Imai, K., Molinaro, G. A. & Ferrone, S. *Transplant Proc.* **12**, 380-383 (1980).
23. Old, L., Stockert, E., Boyse, E. & Kim, J. H. *J. exp. Med.* **127**, 523-539 (1968).
24. Hyman, R., Cunningham, K. & Stallings, V. *Immunogenetics* **10**, 261-271 (1980).
25. Sullivan, A. L., Grasso, J. A. & Weintraub, L. R. *Blood* **47**, 133-143 (1976).
26. Octave, J. N., Schneider, Y.-J., Crichton, R. R. & Trouet, A. *Eur. J. Biochem.* **115**, 611-618 (1981).
27. Greaves, M. & Janossy, G. *Biochim. biophys. Acta* **516**, 193-230 (1978).
28. Brown, J. P. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 539-543 (1981).
29. Metzgar, R. S., Borowitz, M. J., Jones, N. H. & Dowell, B. L. *J. exp. Med.* **154**, 1249-1254 (1981).
30. Bramwell, M. E. & Harris, H. *Proc. R. Soc. B201*, 87-106 (1978).
31. Bramwell, M. E. & Harris, H. *Proc. R. Soc. B203*, 93-99 (1979).

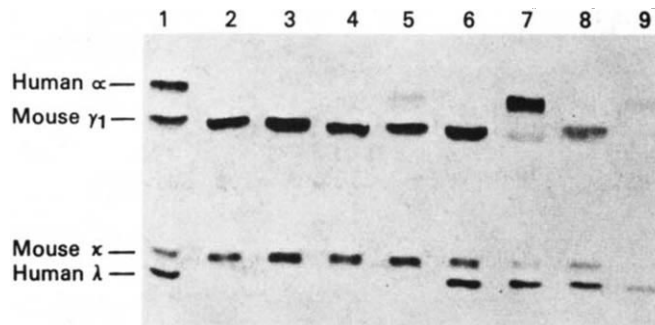
## Assignment of the genes for human $\lambda$ immunoglobulin chains to chromosome 22

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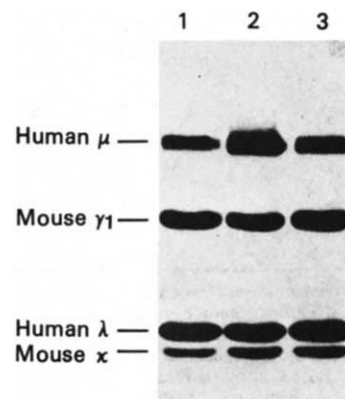
**Human immunoglobulin chains are expressed in somatic cell hybrids between mouse myeloma cells and human B cells<sup>1,2</sup>. By correlating the expression of human immunoglobulin chains and the presence of specific human chromosomes in the hybrid clones, we have assigned the human immunoglobulin heavy-chain gene cluster to human chromosome 14 (refs 1, 2). We have now studied somatic cell hybrids between mouse myeloma cells and either human peripheral lymphocytes or different human  $\lambda$  chain-secreting lymphoblastoid cell lines. Somatic cell hybrid clones were pre-selected for their ability to produce human  $\lambda$  immunoglobulin chains, and studied for the expression of isozyme markers assigned to each of the different human chromosomes. Subclones of human  $\lambda$  chain-secreting hybrid clones were also studied. The results indicate that human chromosome 22 carries the human  $\lambda$  immunoglobulin chain genes.**

We fused hypoxanthine phosphoribosyltransferase (HPRT)-deficient P3 $\times$ 63Ag8 mouse myeloma cells<sup>3</sup> with either peripheral blood lymphocytes or different human lymphoblastoid cells (GM1056 that secretes IgA  $\alpha$ 2,  $\lambda$ ; GM923 that secretes IgA  $\alpha$ 1,  $\lambda$ )<sup>4</sup> to map the chromosomal location of the human  $\lambda$ -chain genes. The hybrids were selected either in hypoxanthine-aminopterin-thymidine (HAT) medium or in HAT medium containing  $10^{-4}$  M ouabain<sup>1,2</sup>. After selection the hybrids were cloned in non-selective medium; each clone derived from an independent hybrid colony. As only one of the alleles for the different immunoglobulin chains is expressed in immunoglobulin-producing cells, the other being silent ('allelic exclusion')<sup>5</sup>, we can predict the presence of hybrids that have retained the chromosomes carrying immunoglobulin chain genes, but do not express them. In fact, we have shown previously that only a fraction of mouse $\times$ human hybridomas containing human chromosome 14 expresses human heavy chains<sup>1,2</sup>. Recently, by using  $\mu$  chain-specific cDNA probes<sup>6</sup> and Southern blotting procedures<sup>6</sup>, we have confirmed assignment



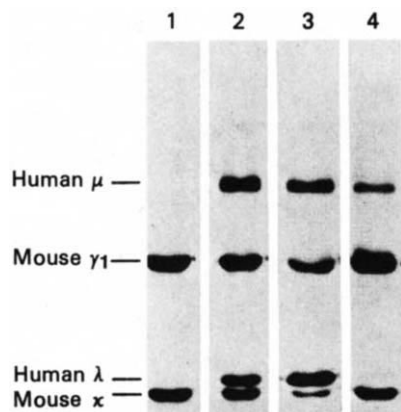
**Fig. 1** SDS-polyacrylamide gel electrophoresis of immunoglobulin chains secreted by somatic cell hybrids between P3 $\times$ 63Ag8 mouse myeloma cells<sup>3</sup> and GM1056 human lymphoblastoid cells<sup>4</sup>. Parent and hybrid cells were grown for 24 h in leucine-deficient media containing  $100 \mu\text{Ci ml}^{-1}$   $^3\text{H}$ -leucine ( $70 \text{ Ci mmol}^{-1}$ ). Cells were pelleted and supernatants (500  $\mu\text{l}$ ) reacted with 25  $\mu\text{l}$  of rabbit anti-human  $\lambda$  chain-specific sera and rabbit anti-mouse immunoglobulins for 1 h in ice. 100  $\mu\text{l}$  of a 10% suspension of fixed *Staphylococcus aureus*<sup>1,2</sup> were added to each reaction mixture for 30 min, or 50  $\mu\text{l}$  of goat anti-rabbit  $\gamma$ -globulin sera were added for 16-18 h, at 4  $^{\circ}\text{C}$ . Double antibody precipitates were collected by centrifugation through a pad of 0.2 ml 1 M sucrose. All immune precipitates were washed with 5% sucrose, 15 mM Tris-HCl pH 7.4, 0.5 M NaCl, 5 mM EDTA, 1% NP-40, 2 mM phenylmethylsulphonyl fluoride. The immune precipitates were resuspended in Laemmli buffer<sup>17</sup> and analysed on 10-11% polyacrylamide gels<sup>17</sup>, embedded in 2,5-diphenyloxazole and subjected to fluorography<sup>18</sup> for 5-14 days. Lanes 2 and 9 show the immunoprecipitates of the culture fluids of P3 $\times$ 63Ag8 and GM1056 respectively. GM1056 cells secrete IgA ( $\alpha$ 2,  $\lambda$ ). The  $\lambda$  chain produced by these cells migrates faster than the mouse  $\kappa$  and the human  $\lambda$  chains produced by independent hybrid clones between P3 $\times$ 63Ag8 cells and human peripheral lymphocytes (see Figs 2, 3). In lanes 1, 3-8 are the immunoprecipitates of P3 $\times$ 63Ag8 $\times$ GM1056 hybrids: human  $\alpha$  and  $\lambda$  chains segregate independently in these hybrids. Immunoprecipitation of cytoplasmic immunoglobulin chains<sup>1,2</sup> present in the hybrids was also carried out, with identical results (data not shown).

of the human heavy-chain genes to human chromosome 14 (T. Dolby, A. Dayton and C. M. C., in preparation). Because of allelic exclusion<sup>5</sup> we decided to pre-select mouse $\times$ human hybridomas for the expression of human  $\lambda$  chains (Figs 1, 2) and to study the  $\lambda$  chain-producing hybrids for the expression of isozyme markers assigned to each of the different human chromosomes. The independent hybrid clones segregated into  $\lambda$  chain-secreting and non-secreting hybrids (Fig. 1). As the parental mouse myeloma cells were secreting immunoglobulins, each of the hybrids producing a human immunoglobulin chain was also secreting it<sup>1,2</sup>.



**Fig. 2** SDS-polyacrylamide gel electrophoresis of immunoglobulin chains secreted by somatic cell hybrids between P3 $\times$ 63Ag8 mouse myeloma cells<sup>3</sup> and human peripheral blood lymphocytes. The human and mouse immunoglobulin chains were labelled and immunoprecipitated as described in Fig. 1 legend. The independent hybrids (lanes 1-3), pre-selected for isozyme analysis, were expressing human  $\lambda$  chain. Immunoprecipitation of cytoplasmic immunoglobulin chains present in the hybrids was also carried out, with identical results (data not shown).

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**Fig. 3** SDS-polyacrylamide gel electrophoresis of immunoglobulin chains secreted by four hybrid subclones of a hybrid clone between P3×63Ag8 cells and human peripheral blood lymphocytes (see Fig. 2). The expression of the human  $\mu$  and  $\lambda$  chains segregated independently in the hybrid subclones. The subclones in lane 1 have lost the ability to secrete (produce) human  $\mu$  and  $\lambda$  chains, whereas those in lanes 2 and 3 have retained the expression of these chains. The subclones in lane 4 have lost the ability to secrete (produce) human  $\lambda$  chains. Immunoprecipitation of cytoplasmic immunoglobulin chains present in the hybrids was also carried out, with identical results (data not shown).

Table 1 shows that only human chromosomes 14 and 22 are likely to carry the human  $\lambda$  heavy-chain genes, as hybrid clones lacking each one of the other human chromosomes were found to produce human  $\lambda$  chains. As the genes for heavy,  $\kappa$  and  $\lambda$  immunoglobulin chains lie on different chromosomes<sup>1,2,7,8</sup>, and

**Table 1** Human chromosomes present in mouse-human hybridomas producing human immunoglobulin  $\lambda$  chains

| Human chromosome | No. of hybridoma clones that are: |     |
|------------------|-----------------------------------|-----|
|                  | +/+                               | +/- |
| 1                | 0                                 | 11  |
| 2                | 0                                 | 11  |
| 3                | 5                                 | 7   |
| 4                | 6                                 | 6   |
| 5                | 9                                 | 3   |
| 6                | 10                                | 10  |
| 7                | 3                                 | 9   |
| 8                | 6                                 | 14  |
| 9                | 0                                 | 12  |
| 10               | 4                                 | 8   |
| 11               | 8                                 | 4   |
| 12               | 2                                 | 10  |
| 13               | 3                                 | 9   |
| 14               | 20                                | 0   |
| 15               | 9                                 | 3   |
| 16               | 2                                 | 10  |
| 17               | 18                                | 2   |
| 18               | 5                                 | 6   |
| 19               | 1                                 | 11  |
| 20               | 4                                 | 8   |
| 21               | 2                                 | 10  |
| 22               | 14                                | 0   |
| X                | 18                                | 2   |

**Table 2** Expression of human  $\lambda$  chains in mouse×human hybridoma subclones

| Human chromosome | No. of hybridoma subclones that are: |     |     |     |
|------------------|--------------------------------------|-----|-----|-----|
|                  | +/+                                  | +/- | -/+ | -/- |
| 1                | 0                                    | 9   | 0   | 7   |
| 2                | 0                                    | 9   | 0   | 7   |
| 3                | 8                                    | 5   | 4   | 3   |
| 4                | 4                                    | 5   | 3   | 4   |
| 5                | 3                                    | 6   | 5   | 2   |
| 6                | 16                                   | 2   | 6   | 1   |
| 7                | 0                                    | 9   | 2   | 5   |
| 8                | 0                                    | 9   | 0   | 7   |
| 9                | 0                                    | 10  | 0   | 7   |
| 10               | 0                                    | 9   | 0   | 7   |
| 11               | 5                                    | 4   | 6   | 1   |
| 12               | 2                                    | 7   | 2   | 5   |
| 13               | 1                                    | 8   | 1   | 6   |
| 14               | 9                                    | 2   | 6   | 1   |
| 15               | 3                                    | 6   | 4   | 3   |
| 16               | 0                                    | 9   | 0   | 7   |
| 17               | 13                                   | 1   | 6   | 1   |
| 18               | 2                                    | 7   | 2   | 5   |
| 19               | 0                                    | 9   | 0   | 7   |
| 20               | 1                                    | 8   | 1   | 6   |
| 21               | 0                                    | 8   | 0   | 8   |
| 22               | 7                                    | 0   | 0   | 7   |
| X                | 5                                    | 2   | 6   | 1   |

+/+, +/−, Same as for Table 1; −/+, clones that do not express human  $\lambda$  chains and carry the numbered chromosome; −/−, clones that neither express human  $\lambda$  chains nor carry the numbered chromosome.

as the heavy-chain genes are located on human chromosome 14 (refs 1, 2), human chromosome 22 is the likely candidate for carrying the human  $\lambda$ -chain genes. All 20 independent hybrid clones tested for nucleoside phosphorylase (a marker of human chromosome 14) were positive for the enzyme as this chromosome is preferentially retained in mouse×human hybridomas<sup>9</sup>. To exclude the possibility that the human  $\lambda$ -chain genes are located on human chromosome 14 and to confirm their assignment to human chromosome 22, we subcloned five independent  $\lambda$  chain-secreting (producing) clones, and studied them for expression of human  $\lambda$  chains (Fig. 3) and of isozyme markers assigned to each of the different human chromosomes. As shown in Table 2, chromosome 14 can be excluded from consideration as two subclones have lost chromosome 14 yet have retained the ability to produce human  $\lambda$  chains. In addition, the results confirm that the genes for  $\lambda$  chains are on human chromosome 22, as this is the only human chromosome consistently present in all subclones secreting human  $\lambda$  chains (Table 2, 2nd column). Loss of human chromosome 22 results in loss of the ability to produce human  $\lambda$  chains (Table 2, 4th column and Fig. 3, lane 4).

Our results parallel those obtained by Southern blotting analysis of hybrid cell DNAs using nucleic acid hybridization probes (W. McBride and P. Leder, personal communication). These findings, in conjunction with the assignment of the heavy-chain gene clusters to human chromosome 14 (refs 1, 2) and of the human  $\kappa$ -chain gene to human chromosome 2 (W. McBride and P. Leder, personal communication), suggest a possible relationship between specific chromosome translocations, immunoglobulin genes and the expression of malignancy in Burkitt's lymphoma<sup>10</sup>. A translocation between chromosomes 8 and 14, described as t(8, 14)(q24; q32), has been found in numerous cases of the disease<sup>11,12</sup>. Recently, two different translocations involving human chromosome 8 have been described in non-African Burkitt's lymphoma<sup>13-15</sup>, which involve the same segment of human chromosome 8 and either human chromosome 22 (that carries the  $\lambda$ -chain genes) or chromosome 2 (that carries the  $\kappa$ -chain gene). More recently, similar translocations, t(2, 8)(p12; q24) and t(8, 22)(q24; q11), have also been found in cases of African Burkitt's lymphoma<sup>16</sup>. These findings indicate that approximately the same segment of human chromosome 8 is translocated to each of the human chromosomes carrying immunoglobulin genes in Burkitt's lymphoma. It would be interesting to determine whether the

Hybrid cells were studied for the expression of isozyme markers assigned to each of the different human chromosomes by starch gel or cellulose acetate gel electrophoresis: chromosomes 1, enolase 1 (EC 4.2.1.11); 2, isocitrate dehydrogenase (EC 1.1.1.42); 3,  $\beta$ -galactosidase (EC 3.2.1.23); 4, phosphoglucomutase 2 (EC 2.7.5.1); 5, hexosaminidase B (EC 3.2.1.30); 6, glyoxalase-1 (EC 4.4.1.5) and phosphoglucomutase 3 (EC 2.7.5.1); 7,  $\beta$ -glucuronidase (EC 3.2.1.31); 8, glutathione reductase (EC 1.6.4.2); 9, aconitase (EC 4.2.1.3); 10, glutamate oxaloacetic transaminase (EC 2.6.1.1); 11, lactate dehydrogenase A (EC 1.1.1.27); 12, lactate dehydrogenase B (EC 1.1.1.27); 13, esterase D (EC 3.1.1.1); 14, nucleoside phosphorylase (EC 2.4.2.1); 15, mannosephosphate isomerase (EC 5.3.1.8); 16, adenine phosphoribosyltransferase (EC 2.4.2.7); 17, galactokinase (EC 2.7.1.6); 18, peptidase A (EC 3.4.11.); 19, glucose phosphate isomerase (EC 5.3.1.9); 20, adenosine deaminase (EC 3.5.4.4); 21, superoxide dismutase 1 (EC 1.15.1.1); 22, arylsulphatase (EC 3.1.6.1); X chromosome, glucose-6-phosphate dehydrogenase (EC 1.1.1.49). +/+, Clones that both produce human  $\lambda$  chains and carry the numbered chromosome; +/-, clones that produce human  $\lambda$  chains and do not carry the numbered chromosome.



break points on human chromosomes 2, 14 and 22 affect chromosomal DNA segments that lie close to or contain the human immunoglobulin chain genes.

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1. Croce, C. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 3416–3419 (1979).
2. Shander, M., Martinis, J. & Croce, C. M. *Transplant Proc.* **12**, 417–420 (1980).
3. Kohler, G. & Milstein, C. *Nature* **256**, 495–497 (1975).
4. Dolby, T. W., DeVuono, J. & Croce, C. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6027–6031 (1980).
5. Early, P. & Hood, L. *Cell* **24**, 1–3 (1981).
6. Southern, E. J. *molec. Biol.* **98**, 503–517 (1975).
7. Hengartner, H., Meo, T. & Muller, E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2883–2887 (1978).
8. D'Eustachio, P., Bothwell, A. L. M., Takaro, T. K., Baltimore, D. & Ruddle, F. H. *J. exp. Med.* **153**, 793–800 (1981).
9. Croce, C. M. *et al. Eur. J. Immun.* **10**, 486–488 (1980).
10. Fialkow, P. J., Klein, G., Gartler, S. M. & Clifford, P. *Lancet* **i**, 384–386 (1970).
11. Manolov, G. & Manolova, Y. *Nature* **237**, 33–34 (1972).
12. Zech, L., Haglund, V., Nilsson, K. & Klein, G. *Int. J. Cancer* **17**, 47–56 (1976).
13. Van Den Berghe, H. *et al. Cancer Genet. Cytogenet.* **1**, 9–14 (1979).
14. Miyoshi, I., Hiraki, S., Kimura, I., Miyamoto, K. & Sato, J. *Experientia* **35**, 742–743 (1979).
15. Berger, R. *et al. Hum. Genet.* **53**, 111–112 (1979).
16. Bornheim, A., Berger, R. & Lenoir, G. *Cancer Genet. Cytogenet.* **3**, 307–316 (1981).
17. Laemmli, U. K. *Nature* **227**, 680–683 (1970).
18. Laskey, R. A. & Bonner, W. M. *Eur. J. Biochem.* **46**, 83–88 (1974).

## Phaseolin mRNA is translated to yield glycosylated polypeptides in *Xenopus* oocytes

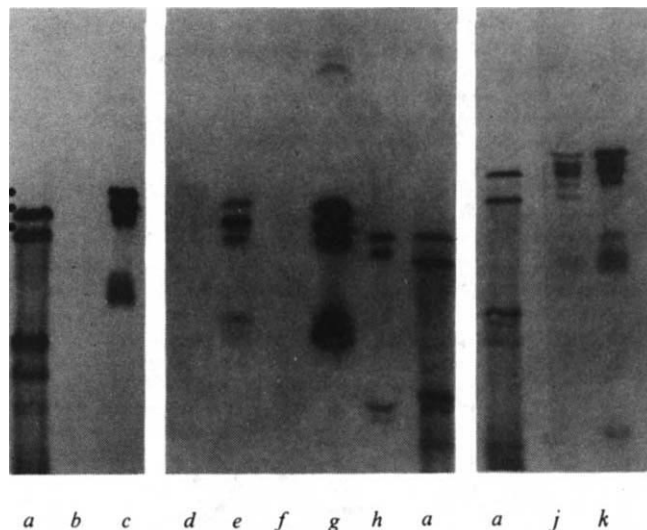
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The cotyledons of developing bean (*Phaseolus vulgaris* L.) seeds rapidly synthesize and accumulate the storage globulin, phaseolin<sup>1</sup>, which seems to be encoded as a small multigene family<sup>2,3</sup>. Phaseolin from the cultivar 'Tendergreen' has been resolved electrophoretically into three components (of differing molecular weights), designated  $\alpha$  (51,000),  $\beta$  (48,000) and  $\gamma$  (45,500). The *in vitro* synthesis of phaseolin has been investigated previously using a cell-free translation system derived from wheat germ<sup>4</sup>. The *in vitro* products did not co-migrate with the authentic polypeptides, the differences apparently being due to the lack of glycosylation of the primary transcripts. We have now used *Xenopus* oocytes to translate mRNA from bean cotyledons and find that phaseolin is synthesized in a glycosylated form very similar to authentic phaseolin and is transported into the membrane fraction of the cells.

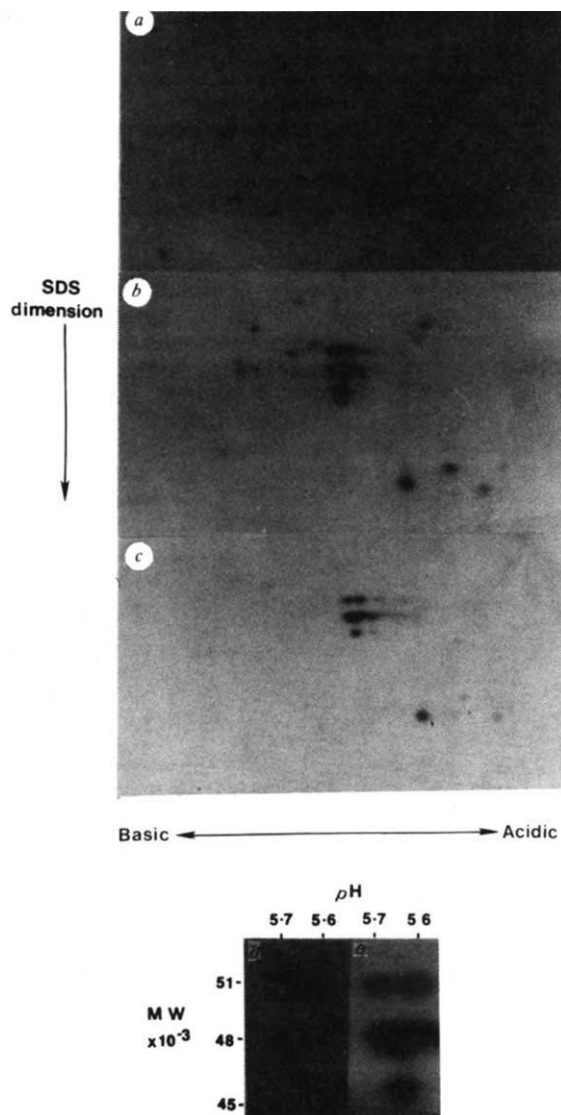
Translation of bean cotyledon mRNA in cell-free extracts from wheat germ<sup>4</sup> yielded polypeptide products that were immunoprecipitated by phaseolin-specific antibodies and which gave peptide maps, using the Cleveland procedure<sup>5</sup>, similar to those of authentic protein<sup>6</sup>. However, SDS-polyacrylamide gel electrophoresis (PAGE) of the *in vitro* products resolved only two bands that migrated close to, but slightly faster than, the positions of the  $\beta$  and  $\gamma$  bands of the native protein<sup>4</sup>. It has previously been suggested that these two bands represent unglycosylated forms of phaseolin, the lower band consisting of co-migrating, unprocessed forms of the  $\beta$  and  $\gamma$  polypeptides and the upper band consisting of unprocessed  $\alpha$  polypeptides. This hypothesis is consistent with the different degrees of glycosylation of the  $\alpha$ ,  $\beta$  and  $\gamma$  polypeptides (3:3:1 ratio of sugar content respectively)<sup>7</sup>.

We have now investigated the hypothesis further by injecting phaseolin mRNA into the cytoplasm of *Xenopus laevis* oocytes and then analysing the translation products of the phaseolin mRNA by one-dimensional SDS-PAGE (Fig. 1). Three major polypeptides were immunoprecipitated with antibody prepared against phaseolin and these migrated at positions closely approximating those of authentic phaseolin (Fig. 1, lane c). In some preparations (Fig. 1, lane k), the  $\beta$  component separated into a closely migrating doublet, possibly representing slight differences in the processing of these polypeptides. The smallest component of the oocyte products migrates with a higher molecular weight (46,500) than that of the authentic protein (45,500). When the glycosylation inhibitor tunicamycin<sup>8</sup> was co-injected with phaseolin mRNA (Fig. 1, lane h), only two immunoprecipitable products were resolved. These co-migrate with the polypeptides synthesized *in vitro* by wheat-germ extracts in the presence of phaseolin mRNA (Fig. 1, lane a). This effect was seen most clearly when glycosylation intermediates (dolichol phosphate) were depleted by incubation of



**Fig. 1** Oocytes were removed from the toad and maintained in modified Barth's solution<sup>15</sup> at 19 °C. Phaseolin mRNA was isolated and translated in the wheat-germ cell-free system<sup>4</sup>. Phaseolin mRNA (20 ng) was injected into isolated oocytes. To study glycosylation inhibition, tunicamycin (40  $\mu\text{g ml}^{-1}$ ) was co-injected with the mRNA, and for such experiments, tunicamycin (2  $\mu\text{g ml}^{-1}$ ) was included in the culture medium. Unless otherwise stated, after 24 h incubation, the oocytes were placed in medium containing 2 mCi ml<sup>-1</sup> <sup>3</sup>H-leucine for a further 24 h. The radiolabelled oocytes were homogenized in 20 mM sodium phosphate pH 7.2, containing 0.5 M NaCl, 1% Nonidet P40 and 1 mM phenylmethylsulphonyl fluoride (40  $\mu\text{l}$  per oocyte). Yolk and pigment were pelleted for 2 min in a microfuge. The homogenate was either analysed directly by SDS-PAGE or immunoprecipitated as described by Sun *et al.*<sup>1</sup>. For subcellular localization studies, oocytes were treated as described elsewhere<sup>8</sup>. The figure shows fluorographs<sup>16</sup> of products separated by SDS-PAGE. The lanes represent: a, total products synthesized in wheat germ in response to phaseolin mRNA; b, immunoprecipitate of mock-injected oocytes; c and k, immunoprecipitate of oocytes injected with phaseolin mRNA; d, immunoprecipitate of the cytosol fraction of oocytes injected with buffer only; e, immunoprecipitate of the cytosol fraction of oocytes injected with phaseolin mRNA; f, as d, membrane fraction of an equivalent amount of oocytes; g, as e, membrane fraction of an equivalent amount of oocytes; h, immunoprecipitate of oocytes co-injected with phaseolin mRNA and tunicamycin and placed in medium containing radioactive amino acids after 24 h incubation; and j, as h, except that injected oocytes were placed immediately in radiolabelled medium. ● Represents the position of stained authentic phaseolin run in the adjacent lane. The specificity of the immunoprecipitation reaction has been demonstrated previously, using the translation products of brome mosaic virus RNA as a control<sup>1</sup>.

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**Fig. 2** Oocyte products, prepared as described in Fig. 1 legend, were separated by two-dimensional gel electrophoresis. *a* Shows the separation of labelled proteins from mock-injected oocytes; *b* shows a similar separation of proteins from oocytes injected with phaseolin mRNA. The proteins in *c* are immunoprecipitates of those in *b* and are shown, enlarged, in *d*. *d* Shows a two-dimensional separation of authentic phaseolin of the cultivar 'Tendergreen'.

the injected oocytes for 24 h before the addition of radioactive amino acids to the medium (Fig. 1, lane *h*). However, when oocytes injected with phaseolin mRNA and tunicamycin were immediately placed in medium containing  $^3\text{H}$ -leucine, both glycosylated and unglycosylated forms of phaseolin were resolved (Fig. 1, lane *j*).

Two-dimensional separation<sup>9,10</sup> of phaseolin polypeptides synthesized in oocytes revealed that they co-migrated precisely with authentic protein in the isoelectric focusing dimension, the  $\alpha$  component separating into two charge isomers. In the SDS-PAGE dimension, the  $\gamma$  component migrates slightly more slowly than the authentic component (Fig. 2). The lower molecular weight components visible in the immunoprecipitate are probably the globulin-2/albumin proteins of *P. vulgaris*<sup>11</sup>. The mRNA preparation codes for these proteins in addition to phaseolin and the antiserum shows some reactivity with them. The identity of the minor high molecular weight products visible in the immunoprecipitates is unknown.

Phaseolin is transported across the endoplasmic reticulum of bean cotyledon cells before its accumulation in the protein

storage bodies<sup>1,12</sup>. Comparison of polypeptides accumulating in the cytosol (Fig. 1, lane *e*) and membrane fractions (Fig. 1, lane *g*) of the oocytes revealed that the bulk of phaseolin was localized in the membrane fraction. The polypeptides present in the cytosol may represent contamination during the isolation procedure; glycosylation probably occurs in the cytosol<sup>8</sup>.

Therefore, phaseolin is synthesized in oocytes as a glycosylated form which virtually co-migrates with authentic phaseolin. The slight difference in size of the  $\gamma$  band may be explained by the fact that, although oocytes can glycosylate primary translation products at the correct site<sup>13</sup>, the attached sugar moiety may not be identical with that attached in the bean cell. Phaseolin is associated with the membrane fraction of oocytes. The storage proteins of corn (zein) have also been synthesized in oocytes in membrane fractions reminiscent of protein bodies<sup>14</sup>. Such observations suggest that these proteins carry signals intrinsic to the formation of the storage organelles characteristic of many proteinaceous seeds. Zein is not glycosylated but is processed by cleavage of a signal sequence<sup>14</sup>. The results reported here argue against the involvement of signal sequence cleavage during the processing of phaseolin because the products synthesized in the presence of tunicamycin, which inhibits glycosylation but does not interfere with sequence cleavage, are the same size as those synthesized in wheat germ. *Xenopus* oocytes are, therefore, a valuable tool with which to investigate the processing of phaseolin.

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1. Sun, S. M., Mutschler, M. A., Bliss, F. A. & Hall, T. C. *Pl. Physiol.*, **Wash.** **61**, 918-923 (1978).
2. Sun, S. M., Slightom, J. L. & Hall, T. C. *Nature* **289**, 37-41 (1981).
3. Brown, J. W. S., Bliss, F. A. & Hall, T. C. *Theor. appl. Genet.* (in the press).
4. Hall, T. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3196-3200 (1978).
5. Cleveland, D. W., Fisher, S. G., Kirschner, M. W. & Laemmli, U. K. *J. biol. Chem.* **252**, 1102-1106 (1977).
6. Ma, Y., Bliss, F. A. & Hall, T. C. *Pl. Physiol.*, **Wash.** **66**, 897-902 (1980).
7. Hall, T. C., McLeester, R. C. & Bliss, F. A. *Pl. Physiol.*, **Wash.** **59**, 1122-1124 (1977).
8. Colman, A. *et al. Eur. J. Biochem.* **113**, 339-348 (1981).
9. O'Farrell, P. H. *J. biol. Chem.* **250**, 4007-4021 (1975).
10. Brown, J. W. S., Ma, Y., Bliss, F. A. & Hall, T. C. *Theor. appl. Genet.* **59**, 83-88 (1981).
11. Brown, J. W. S., Osborn, T. C., Bliss, F. A. & Hall, T. C. *Theor. appl. Genet.* (in the press).
12. Bollini, R. & Chrispeels, M. J. *Planta* **146**, 487-501 (1979).
13. Deacon, N. & Ebringer, A. *FEBS Lett.* **79**, 191-194 (1977).
14. Larkins, B. A., Pedersen, K., Handa, A. K., Hurkman, W. J. & Smith, L. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6448-6452 (1979).
15. Gurdon, J. J. *Embryol.* **36**, 523-540 (1976).
16. Chamberlain, J. P. *Analyt. Biochem.* **98**, 132-135 (1979).

## An 'internal' signal sequence directs secretion and processing of proinsulin in bacteria

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Most secreted proteins, both eukaryotic and prokaryotic, contain an amino-terminal extension that is removed some time during transport (see ref. 1 for a review). The signal hypothesis<sup>2,3</sup> states that these amino-terminal extensions (presequences or signal sequences) serve to bind the protein to the membrane and then to lead the protein through. We recently constructed a series of plasmids in which parts of the *Escherichia coli* prepenicillinase signal sequence<sup>4</sup> were contiguous with the gene for rat preproinsulin, containing 21 of the 24 codons that code for the preproinsulin signal sequence. When *Escherichia coli* was transformed with each of these hybrid signal sequence constructions, an insulin antigen was secreted<sup>5</sup> and correctly



processed to proinsulin<sup>6</sup> in every case. One of these constructions fused the first half of the prepenicillinase signal sequence codons to the preproinsulin gene; as insulin antigen was not secreted when the same codons were fused to the DNA coding only for proinsulin and hence lacking the eukaryotic signal sequence, we hypothesized that the eukaryotic signal sequence was sufficient to direct transport in bacteria. We could not, however, eliminate some minor but specific role for the amino terminus donated by the bacterial signal sequence. One way to do this is to replace the prepenicillinase sequences with that of a nonsecreted *E. coli* protein, for example  $\beta$ -galactosidase. We show here that *E. coli* harbouring a plasmid encoding a  $\beta$ -galactosidase-preproinsulin fusion protein efficiently secretes insulin antigen and processes the protein to proinsulin despite the fact that the signal sequence is internal to the amino terminus.

The plasmid pExlac150<sup>7</sup> is a derivative of pBR322<sup>8</sup> with a *lac* fragment inserted such that unique *EcoRI* and *HindIII* sites directly follow the first eight codons of  $\beta$ -galactosidase and the *lac* UV5 promoter. A *PstI*-terminated cDNA copy of the gene for rat preproinsulin (lacking the first three preproinsulin codons)<sup>9</sup> was inserted into the *HindIII* site of pExlac150 by first removing the 3'-end overhang with the Klenow fragment of *E. coli* DNA polymerase I, then ligating *HindIII* linkers to the blunt ends, cutting with *HindIII* and ligating the fragment to *HindIII*-cut pExlac150. Figure 1 shows the DNA sequence across the region of interest on this construction, called *plac*1947, from the fMet codon of  $\beta$ -galactosidase to the fourth codon of the preproinsulin gene insert (which is the seventh codon of the true preproinsulin gene). There are 18 non-signal sequence amino acids at the amino terminus before the first signal sequence amino acid: 8 are encoded by the first 8 codons of the  $\beta$ -galactosidase gene, 5 by the inserted *EcoRI*-*HindIII* linker, and 5 by the poly-GC tails of the cDNA insert.

*plac*1947 transformed into *E. coli* K-12 strain PR13 was grown in sulphur-deficient medium, with two control constructions in the same *E. coli* strain. These were p241.1947, which encodes preproinsulin and where more than 90% of the insulin antigen is secreted into the periplasmic space, and p241.CB15, which lacks a signal sequence hydrophobic core and where more than 90% of the insulin antigen remains inside the cell, as determined by radioimmunoassay and reported in detail elsewhere<sup>5</sup>. Figure 1 shows the amino acid sequences of the various encoded fusion proteins of *plac*1947, p241.1947 and p241.CB15 and rat preproinsulin, for comparison. Equal

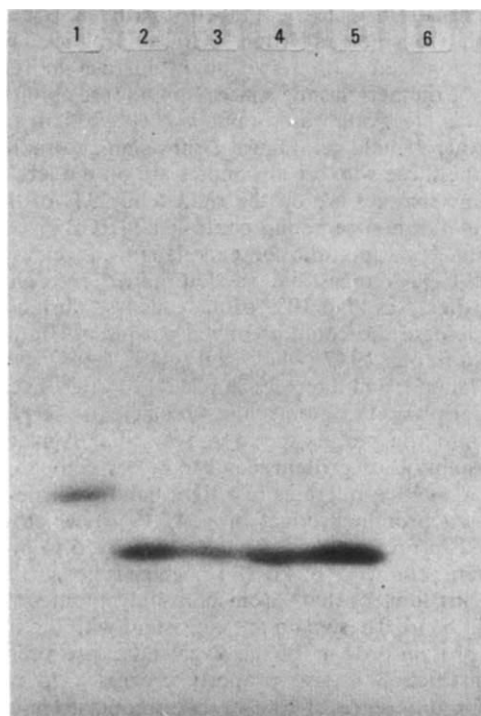
amounts of late log cells were labelled with  $^{35}\text{SO}_4^{2-}$  and either lysed with lysozyme and Triton X-100 to produce a whole cell lysate or digested with lysozyme in sucrose to release the contents of the periplasmic space. Immunoprecipitated insulin antigen was electrophoresed on a 7–15% gradient Laemmli<sup>10</sup> SDS-polyacrylamide gel. Figure 2 shows an autoradiograph of the gel; the three whole-cell samples are on the left, the three periplasmic samples are on the right. Only 9% of the insulin antigen of the non-secreting control, p241.CB15 (whole cell lysate, lane 1; periplasmic contents, lane 6), is released during lysozyme-EDTA digestion, as determined by densitometer tracings; thus, less than 10% of the cells lyse during the treatment to release the contents of the periplasm. Both the new construction, *plac*1947 (whole cell lysate, lane 2; periplasmic content, lane 5), and the control, p241.1947 (whole cell lysate, lane 3; periplasmic content, lane 4), efficiently secrete insulin antigen into the periplasm. Moreover, the  $\beta$ -galactosidase-preproinsulin fusion protein seems to be correctly processed to proinsulin, as it co-migrates in a tight band with the immunoprecipitated protein product of p241.1947, which has been sequenced to prove that it is correctly cleaved to proinsulin<sup>6</sup>. The protein product of p241.CB15, which is not secreted, is 22 amino acids longer<sup>5</sup> than proinsulin and migrates above the proinsulin band. These data are consistent with the hypothesis that the protein product of the  $\beta$ -galactosidase-preproinsulin gene construction is also properly processed to proinsulin, although a difference of one or two amino acids cannot be ruled out.

Our data show that a eukaryotic signal sequence, preceded by 18 amino acids at its amino terminus arising from DNA sequences totally unrelated to any signal peptide, can still direct efficient secretion and also probably correct clipping. Lingappa *et al.*<sup>11</sup> have proposed that ovalbumin contains an internal signal sequence; our experiment supports the concept that the hydrophobic core need not be immediately at the amino terminus to function, although there are limits to the functional placement of the signal sequence. Bedoulle *et al.*<sup>12</sup> have used genetic techniques to fuse most of the gene for a bacterial periplasmic protein, the maltose binding protein, to most of the gene for  $\beta$ -galactosidase.  $\beta$ -Galactosidase enzymatic activity was not detected in the periplasm, but was present in the inner membrane and cytoplasmic fractions<sup>12</sup>. Thus, the instructions for secretion can be overruled if other regions of the protein obscure the presence of a signal sequence or, once secretion is initiated, inhibit completion of secretion.

**Fig. 1** Comparison of the DNA sequence across the fusion region in *plac*1947 and amino acid sequence of the encoded hybrid  $\beta$ -galactosidase-preproinsulin protein product with the amino acid sequences of the three hybrid prepenicillinase-preproinsulin protein products<sup>5</sup>. The DNA sequence begins at the fMet codon of  $\beta$ -galactosidase, contributed by pExlac 150, and ends at the seventh codon of the preproinsulin signal sequence, contributed

|                   |                                                                       |
|-------------------|-----------------------------------------------------------------------|
|                   | MetThrMetIleThrAspSerLeuGluPheGlnAlaTrpGlyGlyGlyGlyGlyTrpMetArgPhe... |
| <i>plac</i> 1947  | ATGACCATGATTACGGATTCACTGGAATTCGAAGCTTGGGGGGGGGGGGGGGGGGTGGATGCCTTC... |
| <i>p lac</i> 1947 | MTMITDSL EFQAWGGGGG WMRFLPLLALLVLWEPKPAQA FVKQ...                     |
| p241.1947         | MSIQHFRVALIP LQGGGGG WMRFLPLLALLVLWEPKPAQA FVKQ...                    |
| p241.CB15         | MSIQHFRVALIP LQR EPKPAQA FVKQ...                                      |
| Preproinsulin     | MALWMRFLPLLALLVLWEPKPAQA FVKQ...                                      |

by a *HindIII*-terminated cDNA insert encoding rat preproinsulin, with the intervening codons (underlined) contributed by an *EcoRI*-*HindIII* linker and the poly-GC tails of the preproinsulin insert. Each line of amino acid sequence represents one continuous sequence which has been grouped from left to right to emphasize similarities and differences as follows: first group,  $\beta$ -galactosidase amino acids; second group, prepenicillinase amino acids; third group, amino acids encoded by DNA linkers and the poly-GC tails of certain preproinsulin gene inserts; fourth group, preproinsulin signal sequence amino acids; fifth group, proinsulin amino acids through the fourth amino acid. The protein product of p241.1947 is effectively secreted by bacteria, where the protein product of p241.CB15 is not secreted at all<sup>5</sup>. A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp. *plac*1947 was constructed as follows: 0.2  $\mu$ g of the *PstI*-terminated cDNA insert from p241.1947<sup>5</sup>, encoding the gene for preproinsulin (but minus the first three codons) and isolated by the methods described elsewhere<sup>15</sup>, was incubated with 1.4 units of *E. coli* DNA polymerase I (Klenow fragment, New England Biolabs) for 2 h at 37 °C in 50  $\mu$ l 10 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 50 mM NaCl, 0.1 mM dCTP. The mixture was phenol extracted, ethanol precipitated and spun in a desk-top clinical centrifuge for 2 min through 2 ml Sephadex G-50 (Pharmacia) in 20 mM NaCl. The ethanol precipitate was resuspended in 10  $\mu$ l 50 mM Tris-HCl pH 7.6, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM ATP, and incubated with 0.2  $\mu$ g of kinased *HindIII* linker (Collaborative Research) and 15 units of T4 DNA ligase (New England Biolabs) overnight at 15 °C. After phenol extraction and ethanol precipitation, the fragment was digested with 35 units *HindIII* (New England Biolabs) for 4 h at 37 °C in 100  $\mu$ l 10 mM Tris pH 7.6, 10 mM MgCl<sub>2</sub>, 1 mM DTT and 50 mM NaCl. The cut fragment was subcloned into *HindIII*-cut pBR322<sup>8</sup> and the isolated *HindIII*-ended 1947 cDNA insert ligated as described above into *HindIII*-cut pExlac 150. The new construction, *plac*1947, was transformed in standard conditions<sup>13</sup> into *E. coli* strain PR13<sup>14</sup>. 10 ml of PR13 harbouring *plac*1947 were grown and the plasmid isolated as described elsewhere<sup>15</sup>. DNA from 2.5 ml of cells was prepared for DNA sequencing exactly as described elsewhere<sup>15</sup> except that the DNA was first digested with *EcoRI* (Bethesda Research Laboratories) and then digested with *RsaI* after radiolabelling. Both labelled fragments were sequenced by the method of Maxam and Gilbert<sup>16</sup>. Sequencing revealed that during the reaction with the Klenow enzyme to blunt-end the *PstI*-terminated 1947 cDNA insert, an extra G was removed from the 5' end of the poly-GC tail, which puts the fragment into frame with pExlac150.



**Fig. 2** Autoradiogram of radiolabelled, immunoprecipitated protein products of three preproinsulin constructions in *E. coli* K-12 strain PR13 fractionated for whole cell contents or for the contents of the periplasm. Lane 1, p241.CB15, whole cell; lane 2, *plac*1947, whole cell; lane 3, p241.1947, whole cell; lane 4, p241.1947, periplasm; lane 5, *plac*1947, periplasm; lane 6, p241.CB15, periplasm. Cells bearing the preproinsulin plasmids were grown in 2YT<sup>17</sup> overnight. 100  $\mu$ l were inoculated into 10 ml S medium<sup>18</sup> supplemented with 10  $\mu$ g ml<sup>-1</sup> thiamine, 40  $\mu$ g ml<sup>-1</sup> L-threonine, and 40  $\mu$ g ml<sup>-1</sup> L-leucine, and grown to an A<sub>550</sub> of 0.7–0.85 at 37 °C. 5 mCi H<sub>2</sub><sup>35</sup>SO<sub>4</sub> (carrier-free, NEN) were added to the cells, which were allowed to grow for a further 10 min, then collected and resuspended in 400  $\mu$ l 50 mM Tris-HCl pH 8, 25% sucrose. The cells were divided into two 200- $\mu$ l aliquots which were both incubated on ice for 15 min with 50  $\mu$ l 10 mg ml<sup>-1</sup> lysozyme in 20 mM EDTA pH 8. A whole-cell fraction was prepared from one of the 200- $\mu$ l aliquots by lysing the cells with 750  $\mu$ l 150 mM Tris-HCl pH 8, 0.2 M EDTA, 2% Triton X-100, and pelleting the cell debris by centrifugation for 1 h at 16,500 r.p.m. in a Sorvall SA-600 rotor. A periplasmic fraction was prepared from the other 200- $\mu$ l aliquot by centrifugation for 20 min at 8,000 r.p.m. in a Sorvall SS-35 rotor and dilution of the supernatant with 750  $\mu$ l of the Triton buffer described above. Both fractions were incubated for 1 h at 37 °C with an amount of anti-insulin serum (an IgG fraction), that is in a 1,000-fold excess over the insulin antigen level in p241.1947 (determined by radioimmunoassay<sup>5</sup>). 100  $\mu$ l of heat killed, formalin-treated *Staphylococcus aureus* (10% v/v, prepared by the method of Kessler<sup>19</sup>) was added, incubated on ice for 30 min and washed by the method of Kessler<sup>19</sup>. The *S. aureus*-radiolabelled antigen complexes were prepared as previously described<sup>5</sup>, except that the samples were resuspended in 50  $\mu$ l sample buffer and electrophoresed on a 7–15% polyacrylamide gradient gel using Laemmli buffers<sup>10</sup> supplemented with 2 mM EDTA. The cells harbouring *plac*1947, p241.1947 or p241.CB15 were grown to A<sub>550</sub> 0.84, 0.76 and 0.74, respectively. Equal whole-cell and periplasmic samples were loaded as follows: *plac*1947, 1  $\mu$ l; p241.1947, 1  $\mu$ l; p241.CB15, 2.5  $\mu$ l. The gel was destained, dried and autoradiographed for 6 h on Kodak X-omat AR-5 film. The film was scanned with an Ortec 4310 densitometer. The actual gel was 20 cm, but only a 3-cm portion is shown here; no other bands appeared in the lanes. By standard liquid radioimmunoassay, described elsewhere<sup>5</sup>, *plac*1947 makes ~1,500 molecules per cell in PR13 (I. Akerblom, personal communication), a whole-cell insulin antigen level similar to that of p241.1947<sup>5</sup>. All manipulations involving cells bearing preproinsulin plasmids were done under P1 containment according to NIH guidelines.

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1. Blobel, G., Walter, P., Chang, C. N., Erickson, A. H. & Lingappa, V. R. *Symp. Soc. exp. Biol.* **33**, 9–36 (1979).
2. Milstein, C., Brownlee, G. G., Harrison, T. M. & Matthews, M. B. *Nature new Biol.* **239**, 117–120 (1972).
3. Blobel, G. & Dobberstein, B. *J. Cell Biol.* **67**, 835–851 (1975).
4. Sutcliffe, J. G. *Proc. natn. Acad. Sci. USA* **75**, 3737–3741 (1978).
5. Talmadge, K., Stahl, S. & Gilbert, W. *Proc. natn. Acad. Sci. USA* **77**, 3369–3373 (1980).
6. Talmadge, K., Kaufman, J. & Gilbert, W. *Proc. natn. Acad. Sci. USA* **77**, 3988–3992 (1980).
7. Weiher, H. thesis, Univ. Heidelberg (1980).
8. Bolivar, F. et al. *Gene* **2**, 95–113 (1977).
9. Villa-Komaroff, L. et al. *Proc. natn. Acad. Sci. USA* **75**, 3727–3731 (1978).
10. Laemmli, U. K. *Nature* **227**, 680–685 (1970).
11. Lingappa, V. R., Lingappa, J. R. & Blobel, G. *Nature* **281**, 117–121 (1979).
12. Bedouelle, H. et al. *Nature* **284**, 78–81 (1980).
13. Mandell, M. & Higa, A. *J. molec. Biol.* **53**, 159–162 (1970).
14. Reiner, A. *J. Bact.* **97**, 1522–1523 (1969).
15. Talmadge, K. & Gilbert, W. *Gene* **12**, 235–241 (1980).
16. Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
17. Miller, J. H. *Experiments in Molecular Genetics*, 433 (Cold Spring Harbor Laboratory, New York, 1972).
18. Roberts, R. B., Abelson, P. A., Cowie, D. B., Bolton, E. T. & Britten, R. J. *Studies of Biosynthesis in Escherichia coli* 5 (Carnegie Institution of Washington Publ. no. 607, Washington DC, 1957).
19. Kessler, S. W. *J. Immun.* **115**, 1617–1624 (1975).

## Point mutation in the TATA box curtails expression of sea urchin H2A histone gene *in vivo*

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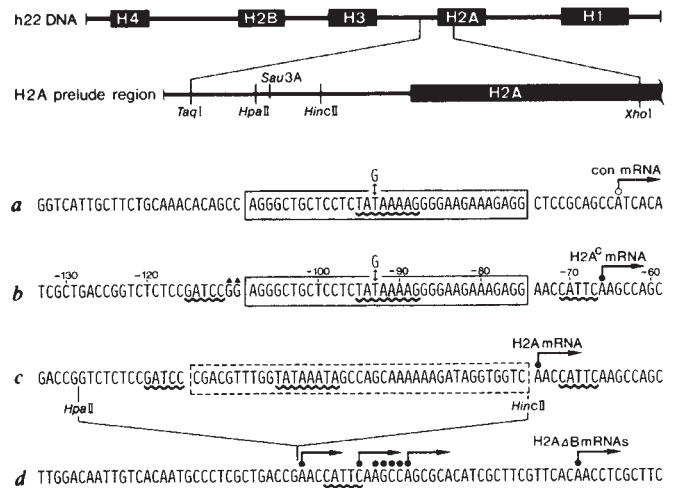
In most, but not all, eukaryotic genes transcribed by polymerase B (II)<sup>1–5</sup>, the site of initiation of transcription is preceded by a TATA<sup>6</sup> sequence<sup>4</sup>, commonly referred to as the Goldberg-Hogness<sup>6</sup> or TATA box, which seems to be required for the generation of faithful 5' termini of mRNAs<sup>7–14</sup>. For all genes except one<sup>9,12,15–17</sup>, deletion of the TATA box reduces the rate of mRNA synthesis<sup>7,10,11,13,18–23</sup>. Point mutations in the TATA box of the chick conalbumin gene diminish transcription *in vitro* 20–40-fold<sup>21,24</sup>. The question remained of whether this effect would persist *in vivo* where far upstream sequences dramatically modulate gene expression<sup>7–11,25,26</sup>. Unfortunately the chicken conalbumin gene is poorly expressed when introduced into eukaryotic cells in culture (B.W., unpublished results) or into *Xenopus* oocytes (S. Swany, personal communication). We have therefore constructed a new gene unit with a chimaeric promoter region by introducing the conalbumin wild-type or mutated TATA box region into the sea urchin H2A histone gene, which is known to be efficiently expressed in *Xenopus* oocytes<sup>27–29</sup>. We report here that with both the wild-type and mutated chimaeric promoters, initiation of transcription occurs at a novel initiation site about 26 nucleotides downstream from the TATA or TAGA boxes, but in the mutant case, the amount of specific transcripts is decreased fivefold.

The scheme for construction of the chimaeric promoter was based on restriction maps and sequencing data of the conalbumin<sup>30</sup> and the H2A histone genes<sup>7,31</sup> (see Fig. 1 legend). This enabled us to assemble a novel gene with a chimaeric promoter which we called H2A<sup>c</sup>. The TATA- and the TAGA-H2A<sup>c</sup> genes were subsequently reintroduced into the 6-kilobase (kb) histone DNA repeat unit<sup>7</sup>, in the place of the wild-type H2A gene. Successful construction of the chimaeras was confirmed at this stage by DNA sequencing<sup>32</sup>. Our sequence manipulations leave the upstream modulator sequences<sup>8</sup> and the initiator

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**Fig. 1** Structure of the 5'-flanking region of the H2A wild-type and mutant genes in the context of the 6-kb histone gene repeat unit. The structural maps of the exchanged DNA segments are shown in the context of the wild-type sequences remaining. **A-D** Depict the nucleotide sequence of the TATA/TAGA region of the chicken conalbumin, the chimaeric TATA/TAGA H2A<sup>c</sup>, the wild-type H2A and the TATA box deletion H2A genes respectively. The exchanged DNA segments are indicated by boxes. The chimaeric promoter mutants contain two extra nucleotides originating from a *Bam*HI linker (▲). Conserved sequences<sup>3,5</sup> are underlined. ●, Map positions of the various H2A mRNA 5' ends (see Fig. 3); ○, the conalbumin mRNA 5' end<sup>30</sup>. The numbering of the TATA-H2A<sup>c</sup> gene refers to the initiation codon of the gene as +1. The chimaeric H2A histone/conalbumin gene promoter mutants were constructed as follows. To replace the TATA box region of the H2A histone gene with the equivalent fragment of the chicken conalbumin gene, we first constructed pBR322-TATA and -TAGA recombinants. Short fragments containing these sequences were prepared from the appropriate mutant and wild-type conalbumin DNAs<sup>21,24</sup> by digestion with *Bst*NI (-44) and *Mbo*II (-9). The ends of each fragment were flush-ended with T4 DNA polymerase. After heat inactivation, <sup>32</sup>P-labelled *Bam*HI linkers were ligated to the flush ends. After *Bam*HI restriction, the DNA fragments terminating in *Bam* sequences were purified and cloned into the *Bam*HI site of pBR322. After amplification, the cloned TATA- and TAGA-containing *Bam*HI fragments were recovered and treated with *Hae*III enzyme to remove the *Bam*-linker sequence at the 3' end of the coding strand. The resulting fragments were ligated to the *Hinc*II-*Xho*I fragment of the H2A gene<sup>7</sup> and the appropriate product selected by gel electrophoresis. The selected DNA fragments were ligated to the 90-bp *Eco*RI-*Sau*3A fragment of the pBR H2A-3 DNA<sup>7</sup> and inserted into an *Eco*RI-*Xho*I-cleaved pBR H2A-3 vector lacking the *Eco*RI-*Xho*I fragment. The *Taq*I-*Xho*I fragments of the resulting recombinants were inserted into the h22 DNA recombinants as described elsewhere<sup>7</sup>.

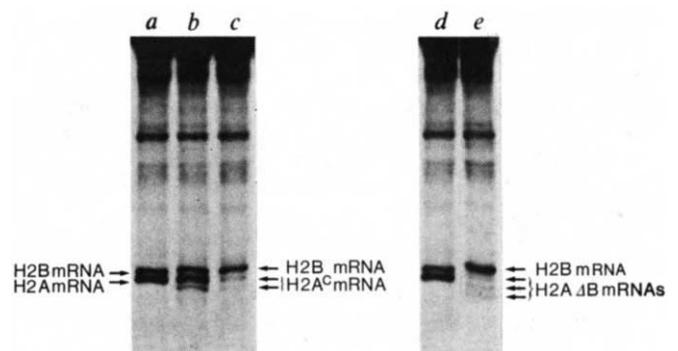


segment<sup>7</sup> untouched. The distance of the modulator from the TATA box differs in the H2A and the chimaeric H2A<sup>c</sup> gene by only 5 nucleotides and between TATA box and initiator segment by 10 nucleotides, as the exchanged TATA box segments were of nearly identical size (see Fig. 1). The circularized repeat unit, free of vector DNA, was injected into *Xenopus* oocyte nuclei together with [ $\alpha$ -<sup>32</sup>P]GTP<sup>33</sup>. Total labelled RNA was isolated and analysed by gel electrophoresis and autoradiography. Expression of the wild-type and mutant H2A genes was monitored in each case against the transcription of the H2B gene which, residing within the same DNA molecule, served as an internal control.

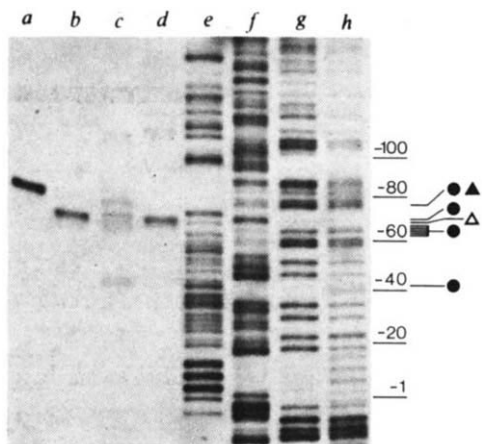
Figure 2b shows that the chimaeric TATA-H2A<sup>c</sup> gene produces a novel H2A<sup>c</sup> mRNA which is resolved into two bands on the electropherogram; the more slowly migrating of these is almost certainly a conformational artefact in this partially denaturing gel system<sup>34</sup> because trimming of the mRNA-DNA hybrids with mung bean nuclease by the modified Berk and Sharp procedure<sup>35,36</sup> produced a single protected DNA band (see Fig. 3b). The TATA-H2A<sup>c</sup> gene is transcribed as rapidly as the wild-type H2A gene (Fig. 2a, b). Calculation of the H2A mRNA synthesis by densitometric tracing of the autoradiogram was performed as described elsewhere<sup>7</sup>. The 5' terminus of the H2A<sup>c</sup> mRNA was mapped with mung bean nuclease<sup>36</sup> to nucleotide position -66 of the TATA-H2A<sup>c</sup> gene (Fig. 3b, e-h) 26 nucleotides downstream of the TATA box (see Fig. 1). In both the wild-type conalbumin and H2A genes, the distance of the initiation site from the TATA box is 28 nucleotides (see Figs 1 and 3, a and b). A hierarchical relationship between the TATA box and the initiator segment<sup>7,8</sup> is again apparent in that the former determines the distance to the initiation site which, however, can be modified by a few nucleotides if a favourable base composition<sup>8</sup> such as CAA or CAT (see Fig. 1) in the sequence ladder is available. The TAGA-H2A<sup>c</sup> gene produces what seems to be an identical transcript to the TATA-H2A<sup>c</sup> mRNA, but at a reduced rate, calculated to be one-fifth of the transcription rate of the TATA-H2A<sup>c</sup> gene (Fig. 2c). A similar reduction in transcription rate is found for the TATA box deletion mutant (Fig. 2e and ref. 7). The 5' terminus of the TAGA-H2A<sup>c</sup> transcript has been mapped to the same nucleotide position (Fig. 3d) as the TATA-H2A<sup>c</sup> mRNA 5' end. This contrasts with the transcripts detected after injection of the TATA box deletion H2A mutant<sup>7</sup> which exhibit a pronounced heterogeneity of 5' ends, as seen in Fig. 3c.

Note that the chicken DNA fragment, although differing in sequence from the substituted sea urchin segment except for the TATA box, can nevertheless replace it functionally. In addition, the natural initiation site, although left untouched, is not used for initiation of transcription of the H2A<sup>c</sup> gene unit, presumably because it is too close to the replaced TATA box. These observations are further evidence for the importance of the TATA box sequence *per se*, and for its predominance in selecting the first nucleotide(s) of the initial transcripts<sup>7,9,12-14,17</sup>.

This work shows the importance of the TATA box for transcription *in vivo*, and particularly of the second T in the sequence. A single base change, from T to G, in the third position of the TATA sequence, does not alter the site of RNA chain initiation, but reduces the rate of transcription by a factor of five. A deletion of the TATA box in the H2A gene also decreased transcription fivefold<sup>7</sup>. The *in vivo* effects of the TAGA box mutation are consistent with the *in vitro* results, where the T to G mutation decreased the rate of transcription



**Fig. 2** Gel analysis of total RNA from microinjected oocytes. Wild-type and mutant 6-kb DNA circles (4 ng per oocyte) were injected together with 0.3  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]GTP<sup>33</sup>. The RNA was extracted and analysed as described elsewhere<sup>7,34</sup>. The labelled endogenous *Xenopus* RNAs are visible in the upper sections of the gel slots. **a-c**, RNA of oocytes injected with h22 wt, TATA-H2A<sup>c</sup>, TAGA-H2A<sup>c</sup> DNA; **d,e**, RNA from h22 wt and h22  $\Delta$ B (TATA box deletion)<sup>7</sup> DNA. The positions of the histone mRNAs are indicated. The amounts of RNA loaded onto each slot correspond to a sample of two oocytes.



**Fig. 3** Mung bean nuclease mapping of the 5' ends of H2A mRNA. For the mapping experiment, total unlabelled RNA from oocytes injected with wild-type and mutant DNAs were purified on CsCl step gradients, hybridized to their cognate 0.9-kb *Hinf*-*Xho*I DNA fragments<sup>31</sup>, 5'-<sup>32</sup>P-labelled at their *Xho*I site (see Fig. 1) and treated with mung bean nuclease as described elsewhere<sup>36</sup>. *a-d*, Labelled DNA fragments protected by RNA from h22 wild-type, TATA-H2A<sup>c</sup>, h22 ΔB (TATA box deletion) and TAGA-H2A<sup>c</sup> templates, respectively. The amount of protected DNA fragments in *a* and *b* correspond to RNA from 0.3 oocytes; for *c* and *d* the amount of DNA fragments loaded onto the slots was increased fivefold to facilitate comparison with *a* and *b*. *e-h* Are the G,A,C,T DNA sequencing reactions<sup>32</sup> of the *Hind*-*Xho*I DNA fragment of TATA-H2A<sup>c</sup> DNA. The numbering of the nucleotide sequence is that of Fig. 1*b*. Symbols at the side denote the uncorrected<sup>7</sup> H2A mRNA 5' map positions: ▲, h22 wild-type; △, TATA and TAGA-H2A<sup>c</sup>; and ●, h22 ΔB (TATA box deletion) mRNAs.

20-fold and did not change the site of RNA chain initiation<sup>21,24</sup>. The less drastic reduction of transcription observed with the TAGA box mutant, relative to the *in vitro* studies, may be due to the activity of the modulator sequences in the *in vivo* system. Almost all the 5' termini of the TAGA-H2A<sup>c</sup> transcripts can be mapped to a single (or at least predominant) initiation site with few, if any, transcripts initiating further upstream, while transcripts of TATA-deletion mutants in H2A and other genes quite frequently show a plurality of 5' termini<sup>7,9-12</sup>. If, as speculated, the TATA box serves to guide the RNA polymerase into a correct initiation frame<sup>7,13</sup>, a T to G mutation may simply promote a less productive 'initiation complex'. Because such a complex may still represent, energetically, the most favoured structure, alternative starting points, readily detected in TATA-deletion mutants, cannot arise.

Some eukaryotic genes known to be expressed at high rate, such as viral<sup>1,4,37</sup> genes and the predominant H4 gene of two sea urchins<sup>5</sup>, do not have recognizable or very deformed TATA boxes. It will be of interest to learn what controls initiation of transcription in these cases.

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1. Ziff, E. B. & Evans, R. M. *Cell* **15**, 1463-1475 (1978).
2. Gannon, F. *et al. Nature* **278**, 428-434 (1979).
3. Busslinger, M., Portmann, R., Irminger, J. C. & Birnstiel, M. L. *Nucleic Acids Res.* **8**, 957-977 (1980).
4. Breathnach, R. & Chambon, P. A. *Rev. Biochem.* **50**, 349-384 (1981).
5. Hentschel, Ch. & Birnstiel, M. L. *Cell* **25**, 301-313 (1981).
6. Goldberg, M. thesis, Stanford Univ. (1979).
7. Grosschedl, R. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1432-1436 (1980).
8. Grosschedl, R. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7102-7106 (1980).
9. Benoist, Ch. & Chambon, P. *Nature* **290**, 304-310 (1981).
10. Faye, G., Leung, D. W., Tatchell, K., Hall, B. D. & Smith, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2258-2268 (1981).

11. Dierks, P., van Ooyen, A., Mantei, N. & Weissmann, C. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1411-1415 (1981).
12. Mathis, D. J. & Chambon, P. *Nature* **290**, 310-315 (1981).
13. Corden, J. *et al. Science* **209**, 1406-1414 (1980).
14. Sassone-Corsi, P., Corden, J., Kedinger, C. & Chambon, P. *Nucleic Acids Res.* **9**, 3941-3958 (1981).
15. Gluzman, Y., Sambrook, J. F. & Frisque, R. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3898-3902 (1980).
16. Benoist, C. & Chambon, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3865-3869 (1980).
17. Ghosh, P. K., Lebowitz, P., Frisque, R. J. & Gluzman, Y. *Proc. natn. Acad. Sci. U.S.A.* **78**, 100-104 (1981).
18. Wasyluk, B., Kedinger, C., Corden, J., Brison, O. & Chambon, P. *Nature* **285**, 267-373 (1980).
19. Hu, S. L. & Manley, J. L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 820-824 (1981).
20. Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. *Proc. natn. Acad. Sci. U.S.A.* **78**, 879-883 (1981).
21. Wasyluk, B. & Chambon, P. *Nucleic Acids Res.* **9**, 1813-1824 (1981).
22. McKnight, S. & Gavis, E. *Nucleic Acids Res.* **8**, 5931-5948 (1980).
23. Osborne, T. F., Schell, R. E., Burch-Jaffe, E., Berget, S. J. & Berk, A. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1381-1385 (1981).
24. Wasyluk, B. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 7024-7028 (1980).
25. Gruss, P., Dhar, R. & Khoury, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 943-947 (1981).
26. Guarente, L. & Ptashne, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2199-2203 (1981).
27. Kressmann, A., Clarkson, S. G., Telford, J. L. & Birnstiel, M. L. *Cold Spring Harb. Symp. quant. Biol.* **42**, 1077-1082 (1977).
28. Probst, E., Kressmann, A. & Birnstiel, M. L. *J. molec. Biol.* **135**, 700-732 (1979).
29. Hentschel, Ch., Probst, E. & Birnstiel, M. L. *Nature* **288**, 100-102 (1980).
30. Cochet, M. *et al. Nature* **282**, 567-574 (1979).
31. Schaffner, W. *et al. Cell* **4**, 655-671 (1978).
32. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).
33. Kressmann, A. & Birnstiel, M. L. *NATO Adv. Study Inst. Ser.* **31**, 383-407 (1980).
34. Gross, K., Probst, E., Schaffner, W. & Birnstiel, M. L. *Cell* **8**, 455-469 (1976).
35. Weaver, R. F. & Weissmann, C. *Nucleic Acids Res.* **7**, 1175-1193 (1979).
36. Green, M. R. & Roeder, R. G. *Cell* **22**, 231-242 (1980).
37. Baker, C. C., Herisse, G., Courtois, G., Galibert, F. & Ziff, E. *Cell* **18**, 569-580 (1979).

## Radiation-induced base substitution mutagenesis in single-stranded DNA phage M13

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**Radiation-induced mutagenesis of *Escherichia coli* depends on the ability of the bacteria to perform error-prone repair<sup>1,2</sup>. This SOS repair activity is induced by irradiation of bacteria and gives rise to mutations both at the site of radiation-induced lesions (targeted mutations) and in undamaged DNA (untargeted mutations)<sup>1,3-6</sup>. To elucidate the relative contributions of targeted and untargeted mutations to  $\gamma$  and UV radiation mutagenesis we have determined the DNA sequences of 174 M13 revertant phages isolated from stocks of irradiated or unirradiated amber mutants grown in irradiated (SOS-induced) or unirradiated (non-induced) host bacteria. As reported here, differences in the spectra of base change mutations induced in the various conditions were apparent, but we detected no obvious specificity of mutagenesis. In particular, in our conditions, pyrimidine dimers did not seem to be the principal sites of UV-induced base substitution mutagenesis, suggesting that such mutagenesis occurs at the sites of lesions other than pyrimidine dimers, or is untargeted.**

We used single-stranded DNA phage M13 to study independently the effects of target DNA irradiation and the consequences of induction of the SOS repair system. M13 is particularly useful in such an investigation because (1) mutagenesis of single-stranded DNA phages by UV and  $\gamma$

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**Table 1** Survival and mutagenesis of irradiated and unirradiated M13 grown in UV-irradiated and unirradiated bacteria

|              | Irradiation | Phage Host | No. of expts | Survival                      | Mutation frequency            |
|--------------|-------------|------------|--------------|-------------------------------|-------------------------------|
| <i>am6H1</i> | 0           | 0          | 2            | —                             | $1.6(\pm 0.7) \times 10^{-4}$ |
|              |             | UV         | 2            | —                             | $1.3(\pm 0.7) \times 10^{-3}$ |
|              | UV          | 0          | 2            | $3.4(\pm 2.6) \times 10^{-4}$ | $1.6(\pm 0.3) \times 10^{-4}$ |
|              |             | UV         | 2            | $5.7(\pm 1.0) \times 10^{-4}$ | $2.6(\pm 0.9) \times 10^{-3}$ |
| <i>am7H3</i> | 0           | 0          | 5            | —                             | $7.4(\pm 3.3) \times 10^{-6}$ |
|              |             | UV         | 5            | —                             | $3.8(\pm 2.7) \times 10^{-5}$ |
|              | UV          | 0          | 2            | $1.7(\pm 0.5) \times 10^{-2}$ | $7.7(\pm 0.6) \times 10^{-6}$ |
|              |             | UV         | 2            | $2.8(\pm 0.5) \times 10^{-2}$ | $1.5(\pm 0.4) \times 10^{-4}$ |
|              | $\gamma$    | 0          | 5            | $4.7(\pm 1.8) \times 10^{-3}$ | $3.0(\pm 1.0) \times 10^{-5}$ |
|              |             | UV         | 5            | $7.1(\pm 3.5) \times 10^{-3}$ | $3.5(\pm 3.1) \times 10^{-4}$ |

log phase cultures of KA798 and KA805 *SupD* grown with bubbling at 32 °C to an  $A_{560} = 0.3$  were divided into two portions, one of which was irradiated with UV light. The cultures were grown in the dark for a further 20 min to maximize SOS induction<sup>4</sup> and were then infected with intact or irradiated M13 phage. After adsorption at room temperature for 3 min the infected bacteria were plated out and incubated overnight at 37 °C. Plaques were counted and revertants picked for DNA sequence analysis. In experiments with *am6H1* phage, the UV dose to phage was 90 J m<sup>-2</sup> and to the host bacteria 50 J m<sup>-2</sup>. In experiments with *am7H3* phage, phage were irradiated with 60 J m<sup>-2</sup> and the host bacteria with 60 J m<sup>-2</sup>. The  $\gamma$ -ray dose (in broth in air) was 1.5 Mrad. Mutation frequencies are the proportion of revertant phages (titled on KA798) to total phages (titled on KA805 *SupD*); standard deviations are given in parentheses.

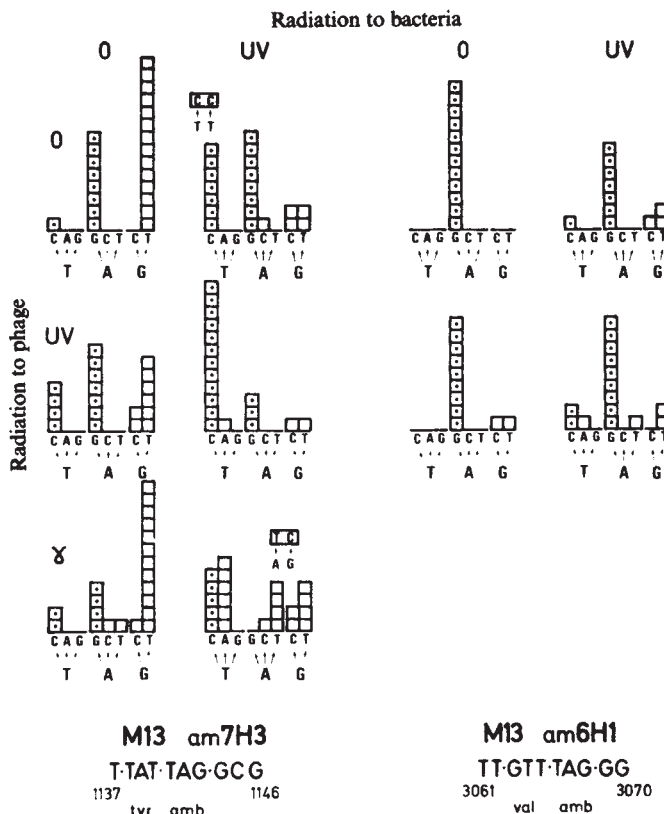
irradiation is dependent on the activity of the same *recA*<sup>+</sup> and *lexA*<sup>+</sup> bacterial genes that are required for the induction of all SOS functions in *E. coli*, including radiation mutagenesis<sup>5,6</sup>, (2) its single-stranded DNA is not subject to error-free repair by excision<sup>7</sup> or by multiplicity reactivation (recombination repair)<sup>8</sup>, and (3) its DNA has been completely sequenced<sup>9</sup> and, as it is single stranded, it is possible unambiguously to identify the exact bases involved in any sequence alteration.

Forty-nine revertants of *am6H1* and 125 revertants of *am7H3* were isolated from populations of irradiated and unirradiated phages grown in irradiated and unirradiated bacteria (Table 1), and the DNA sequence in the region of the amber codon was determined by the Sanger method<sup>9,10</sup>. Figure 1 shows the number of occurrences of each reversion sequence. Except for the TAG → GAG transversion, which was not found for either amber, all other detectable single-nucleotide changes were observed. (TAG → TAA produces an ochre nonsense codon which would not have been detected as a revertant.) Our failure to detect any GAG revertants might have been due to the infrequency of the T → G transversion or the inability of glutamic acid to substitute for glutamine at these two sites.

The limited number of mutants that can be analysed by DNA sequencing imposes a limit on the significance of differences observed between the spectra of base changes induced by various treatments. An additional problem is that pre-existing mutants may make a significant contribution to the spectra for spontaneous revertants and revertants isolated after those treatments which result in reversion frequencies close to the spontaneous frequency. (Reconstruction experiments showed no significant growth advantage or disadvantage of revertants.) In an attempt to minimize the problems of pre-existing revertants, several stocks of the amber phages were prepared from isolated plaques for each experiment. For those treatments resulting in an elevated reversion frequency, the fact that the revertants were isolated from infective centres ensures that they arose independently, and are therefore more likely to represent accurately the mutational spectrum.

In addition to increasing the frequency of mutations of unirradiated phage, UV irradiation of host cells increased the variety of base changes. Some of the base changes found among these untargeted mutants had not been detected in the spon-

taneous spectra, while some of the base changes found frequently in the spontaneous spectra were less common in the untargeted spectra. This finding agrees with reports that in addition to increasing the frequency of mutation of unirradiated and irradiated phage, induction of the SOS repair system may alter the specificity of mutagenesis<sup>6,11</sup>. No increase in the mutation frequency was observed when UV-irradiated phage were grown in unirradiated hosts, but a considerable increase was observed when they were grown in UV-irradiated hosts (Table 1)<sup>3,4,6</sup>. This implies that UV photoproducts in target M13 DNA are not directly mutagenic, but that they are mutagenic when the SOS repair system has been induced. To assess the role of pyrimidine dimers in this mutagenesis we had chosen to study two amber mutants in which the thymine had the potential to be dimerized with a neighbouring thymine in the preceding codon. (Furthermore, in the case of the *am6H1* mutant the third base of the preceding valine codon could be replaced by any other nucleotide without changing the sense of this codon.) Few of the UV-induced mutants of *am6H1* involved the thymine. On the other hand, the T → C transition seemed to make a major contribution to targeted mutagenesis of *am7H3*, but this transition also contributed significantly to untargeted mutagenesis of this mutant. The fact that UV-induced mutations frequently involved the adenine and guanine bases of both amber mutants suggests that pyrimidine dimers are not the main sites of base substitution mutagenesis in UV-irradiated M13 DNA. Two alternative possibilities are that photoproducts other than pyrimidine dimers are important targets for UV-induced base substitution mutagenesis or that pyrimidine dimers can have distal mutagenic effects on the same molecule.



**Fig. 1** DNA sequences of revertants of irradiated and unirradiated M13 *am7H3* and *am6H1* grown in UV-irradiated and unirradiated bacteria. Revertants from the experiments shown in Table 1 were isolated and their DNA sequences in the region of the amber mutation determined by the Sanger method<sup>9,10</sup>. For *am6H1* the M13 *HhaI* fragment 5 (ref. 19) was used as primer, and for *am7H3* the M13 *SalI* fragment 1 (ref. 19) was used. After extension by *E. coli* DNA polymerase I, Klenow subfragment, the primers were removed by *HhaI* digestion and the products analysed on 12% polyacrylamide gels. Each box corresponds to an independently isolated revertant: □, transition; □, transversion; ☒, tandem base changes.

$\gamma$ -Induced mutagenesis of *E. coli* depends on the *recA*<sup>+</sup> and *lexA*<sup>+</sup> gene functions, but seems less dependent on SOS-induced *de novo* protein synthesis<sup>2</sup>. Consistent with this finding,  $\gamma$  irradiation of *am7H3* was mutagenic even when the phage was grown in unirradiated hosts. However, growth of  $\gamma$ -irradiated phage in UV-irradiated hosts increased mutant yields considerably, suggesting that  $\gamma$  irradiation, like UV irradiation, also causes DNA damage whose mutagenic potential is only realized in SOS-induced cells<sup>12</sup>. Insofar as the spectra of base changes induced by  $\gamma$  and UV irradiation differ (difficult to assess on the small sample shown here, but see refs 11, 13, 14), these lesions are likely to be different, a predictable conclusion in view of the different DNA lesions caused by UV and  $\gamma$  irradiation<sup>15</sup>.

Other investigators (ref. 13 and refs therein) have found that UV light sometimes induces tandem base change mutants whose appearance has been considered as evidence of mutations occurring at the sites of pyrimidine dimers<sup>1,5,13</sup>. In our experiments two such mutants were found in phage grown in UV-irradiated hosts, but neither of these could be attributed to targeted mutations at the sites of pyrimidine dimers, as one was from unirradiated phage and the other involved two purines. These mutants, and the fact that both UV and  $\gamma$  irradiation induced a wide variety of different base changes in M13 (Fig. 1) and in the *lac i* gene of *E. coli*<sup>13,14</sup>, raise the possibility that induction of the SOS repair system, by UV or  $\gamma$  radiation, causes clusters of targeted and untargeted base changes. The observation that non-tandem double base changes in the anticodon sequences of an *E. coli* tRNA gene occur at much higher frequencies after UV irradiation than expected from the frequency of single base mutants<sup>16</sup> supports this idea.

The contribution of untargeted mutagenesis to UV mutagenesis may be higher for phage genomes than for bacterial genomes, because phage DNA experiences more rounds of replication in the presence of SOS mutator activity<sup>1</sup> and because post-replicative mismatch correction<sup>17</sup> of untargeted mutations may be less efficient for phage DNA. Our data indicate that, in the conditions used, thymine dimers are not the principal sites of UV-induced base substitution mutagenesis of phage M13. If this is a general phenomenon, the photoreversibility of the mutagenic effects of UV on bacteria<sup>18</sup> and the photoreversibility of the UV induction of the SOS repair system<sup>3</sup> may indicate that pyrimidine dimers are primarily inducing lesions for a process (SOS repair) necessary for targeted and leading to untargeted mutagenesis.

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1. Witkin, E. M. & Wermundsen, I. E. *Cold Spring Harb. Symp. quant. Biol.* **43**, 881-886 (1979).
2. Bridges, B. A. & Mottershead R. P. *Mutat. Res.* **52**, 151-159 (1978).
3. Weigle, J. J. *Proc. natn. Acad. Sci. U.S.A.* **39**, 628-636 (1953).
4. Defais, M., Caillet-Fauquet, P., Fox, M. S. & Radman, M. *Molec. gen. Genet.* **148**, 125-130 (1976).
5. Caillet-Fauquet, P., Defais, M. & Radman, M. *J. molec. Biol.* **117**, 95-112 (1977).
6. Bleichrodt, J. F. & Verheij, W. S. D. *Molec. gen. Genet.* **135**, 19-27 (1974).
7. Yarus, M. & Sinsheimer, R. L. *Biophys. J.* **7**, 267-278 (1967).
8. Tessaun, E. S. & Ozaki, T. *Virology* **12**, 431-449 (1960).
9. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
10. Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. *J. molec. Biol.* **143**, 161-178 (1980).
11. Bleichrodt, J. F. & Roos-Verheij, W. S. D. *Molec. gen. Genet.* **176**, 155-160 (1979).
12. Bridges, B. A. *Molec. gen. Genet.* **151**, 115-120 (1977).
13. Coulondre, C. & Miller, J. H. *J. molec. Biol.* **117**, 577-606 (1977).
14. Glickman, B. W., Rietveld, K. & Aaron C. S. *Mutat. Res.* **69**, 1-12 (1980).
15. Wang, S. Y. (ed.) *Photochemistry and Photobiology of Nucleic Acids* Vol. 2 (Academic, New York, 1976).
16. Coleman, R. D., Dunst, R. W. & Hill, C. W. *Molec. gen. Genet.* **177**, 213-222 (1980).
17. Radman, M. et al. *Cold Spring Harb. Symp. quant. Biol.* **43**, 937-946 (1979).
18. Witkin, E. M. *Science* **152**, 1345-1353 (1966).
19. Van Zezebeek, P. M. G. F., Hulsebos, T. J. M. & Schoenmakers, J. G. G. *Gene* **11**, 129-148 (1980).

## Preferential cleavage of phage $\lambda$ repressor monomers by *recA* protease

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**Expression of phage lytic functions in  $\lambda$  lysogens of *Escherichia coli* is prevented by phage repressor, which binds to specific operator sequences on the prophage DNA. Phage induction accompanies the expression of an entire set of 'SOS functions', which occur when host DNA is damaged or DNA synthesis is interrupted. In these conditions,  $\lambda$  repressor is thought to be inactivated by *recA* protease, thereby leading to expression of phage lytic functions<sup>1</sup>. We have now characterized the repressor from a  $\lambda$  mutant, *ind*<sup>s</sup>-1, which, as prophage, is more easily induced than wild-type phage by a weak inducing treatment<sup>2</sup>. At high concentrations, *ind*<sup>s</sup>-1 repressor differs from wild-type repressor in two ways: whereas wild-type repressor forms dimers and becomes relatively resistant to the *recA* protease, a much greater proportion of *ind*<sup>s</sup>-1 repressor remains monomeric and the protein remains sensitive to the protease. These findings support previous indications<sup>3</sup> that repressor monomers are the preferred substrate for protease, both *in vivo* and *in vitro*.**

Phage  $\lambda$  repressor has two functional and physical domains: an amino-terminal one determining operator recognition and a carboxy-terminal one determining the formation of repressor oligomers<sup>4</sup>. As only dimers of repressor bind to phage operators with high affinity and specificity<sup>4,5</sup>, separation of the two domains by proteolytic cleavage should inactivate the protein, and indeed, the purified N-terminal fragment binds to operator DNA with 10<sup>4</sup>-fold lower affinity than does intact repressor<sup>4</sup>. *In vivo* and *in vitro* the *recA* protease specifically cleaves repressor into the two domains, thereby inactivating it<sup>1,6</sup>.

Two lines of evidence suggest that  $\lambda$  repressor dimers are relatively resistant to the *recA* protease. *In vitro*, the fraction of repressor cleaved, as measured by inactivation of repressor DNA binding activity, decreases at repressor concentrations where a significant proportion of repressor exists as dimers<sup>3</sup>. This effect is not due to saturation of the protease, because the rate of cleavage of admixed P22 repressor is unaffected even by high levels of  $\lambda$  repressor<sup>3</sup>. *In vivo*, induction of  $\lambda$  is deficient in a cell which overproduces repressor, whereas the heteroimmune phage  $\lambda$  imm<sup>434</sup>, present as a prophage, is normally inducible in such cells<sup>7</sup>.

To test further whether repressor dimers are protease resistant, we have characterized the repressor of the hyperinducible mutant, *ind*<sup>s</sup>-1. Because this mutation lies in the region of *cI*, which encodes the C-terminus of repressor (see Fig. 1), we thought that *ind*<sup>s</sup>-1 repressor might be altered in its ability to dimerize and might therefore have increased susceptibility to *recA* protease at high concentrations.

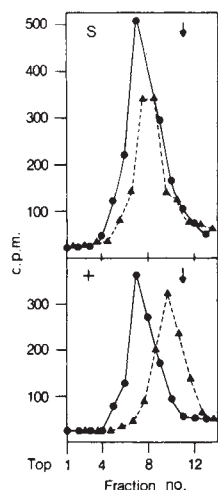
Two *in vivo* tests argue against the possibility that *ind*<sup>s</sup>-1 induces more readily because its repressor is defective in operator binding: (1) the mutant can form lysogens as efficiently as *ind*<sup>+</sup> phage<sup>8</sup>; and (2) while the rate of spontaneous phage release from a *ind*<sup>s</sup>-1 lysogen of a *recA*<sup>+</sup> host is an order of magnitude higher than for *ind*<sup>+</sup>, there is no spontaneous phage production by the mutant prophage in a *recA*<sup>-</sup> host, which lacks *recA* protease activity (unpublished observations). We conclude that the *ind*<sup>s</sup>-1 repressor is functionally stable *in vivo* and likely to be altered in its interaction with the protease.

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**Fig. 1** Sedimentation of  $\lambda ind^+ - 1$  and  $\lambda ind^+$  repressors.  $^{35}S$ -labelled repressor was diluted to  $4 \times 10^{-9}$  M in a buffer containing 0.01 M PIPES pH 6.9, 0.2 M NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 50  $\mu g ml^{-1}$  bovine serum albumin and 10  $mg ml^{-1}$  bovine haemoglobin marker ( $\downarrow$ ), either with ( $\Delta$ ) or without ( $\bullet$ ) unlabelled repressor at  $10^{-6}$  M. Following 90 min incubation at  $0^\circ C$  to establish the monomer-dimer equilibrium, 0.3 ml of each sample was layered on a 5-ml 5–20% sucrose gradient in the same buffer<sup>9</sup>. Gradients were centrifuged in a Beckman SW60Ti rotor for 20 h at 59,000 r.p.m. at  $4^\circ C$ , 0.3-ml fractions were collected, the position of the haemoglobin marker determined, haemoglobin added to each fraction up to this same peak level to standardize the counting buffer and the radioactivity of each fraction

measured. Bottom panel,  $ind^+$ ; top panel,  $ind^+ - 1$ . To determine the position of  $ind^+ - 1$  on the  $\lambda$  genetic map,  $\lambda ind^+ - 1$  *sus* 029 was crossed with various  $\lambda spi^-$  mutants with deletions which end in *cl* or left of *cl* (ref. 11). Because  $spi^-$  phage fail to grow on a *recA^-* host,  $sus^+ spi^-$  phage produced by recombination between the right end of the *spi* deletion and *sus*  $0^+$  could be selected by plaque formation on *recA^- sup^+* bacteria. At least 1,000 of these recombinants were scored for the presence of  $ind^+ - 1$  by their plaque type on DM1285, on which  $ind^+$  and  $ind^+ - 1$  phage form turbid and clear plaques respectively<sup>12</sup>. These crosses established that  $ind^+ - 1$  lies between the right ends of *spi-23* and *spi-91* (ref. 11), placing it in the left one-third of *cl* or in the right end of *rex*. This map location was verified by *in vitro* recombination. To facilitate purification of  $ind^+ - 1$  repressor, plasmid pKB280 (ref. 13) was modified to overproduce  $ind^+ - 1$  mutant repressor. A 520-base pair *Hind*III fragment, which includes the left one-third of the *cl* gene, was removed from pKB280 to make pBK1; the homologous fragment excised from phage  $\lambda ind^+ - 1$  was then inserted into pBK1 to produce pBK2. pBK1, which had the portion of *cl* coding for the  $NH_2$ -terminus of repressor, was resistant to  $\lambda cl^-$  mutants (see also ref. 5). However, pBK1 was sensitive to  $\lambda vir$ . Therefore, cells carrying pBK2 could be selected on the basis of their resistance to  $\lambda vir$ . The presence of  $ind^+ - 1$  on pBK2 was confirmed by rescue of  $ind^+ - 1$  from pBK2 by genetic recombination. Repressor levels in cells carrying pBK2 were lowered by introducing into them *F' lacI<sup>Q1</sup>* which overproduces *lac* repressor<sup>14</sup>. Because the phage repressor genes in pKB280 and pBK2 are fused to the *lacZ* promoter-operator region, the *lac* repressor reduced the phage repressor levels. After further inducing treatment with mitomycin C, it became possible to superinfect cells with  $\lambda c47$ , which has a clear-plaque mutation near  $ind^+ - 1$ , and thereby to rescue the  $ind^+ - 1$  alleles by phage-plasmid recombination. Phage carrying  $ind^+ - 1$  were distinguished from  $\lambda c47$  by production of a turbid plaque on normal indicator C600 and a clear plaque on DM1285, as described previously<sup>12</sup>. Repressor was purified from extracts of cells carrying pKB280 or pBK2 by method (1) of Johnson *et al.*<sup>15</sup> and was estimated to be 98% pure by gel electrophoresis. The mobility of the purified  $ind^+ - 1$  repressor in polyacrylamide gels was slightly greater than that of  $ind^+$  (see Fig. 2). By the filter binding assay, the DNA binding activity of one of our preparations of  $ind^+ - 1$  repressor was found to be twofold less than wild-type repressor purified by the same procedure. While this effect may have resulted from a change in dimer association as described in the text, variability in the same range can be found in different preparations of wild-type repressor<sup>10</sup>. Repressor concentrations throughout are given in monomers.

Plasmid pBK2, which overproduces  $ind^+ - 1$  repressor, was isolated and used as a source of mutant repressor (see Fig. 1) to be compared with wild-type repressor for its ability to form oligomers and to serve as substrate for *recA* protease. The repressor concentration in these experiments was in the range  $4 \times 10^{-9}$ – $1.4 \times 10^{-6}$  M, which was achieved by adding varying quantities of unlabelled repressor to a small quantity of  $^{35}S$ -labelled repressor and incubating for a period of time to allow establishment of equilibrium between monomers and higher oligomeric forms of repressor. Results were analysed by monitoring the labelled repressor. Oligomer formation was compared by measuring sedimentation rates in sucrose gradients.

Wild-type repressor sedimented faster at  $10^{-6}$  and  $10^{-7}$  M than at  $4 \times 10^{-9}$  M, as expected<sup>9</sup>, indicating oligomerization at the higher concentrations (Fig. 1 and data not shown). By contrast, the sedimentation rate of  $ind^+ - 1$  repressor did not increase significantly except at the highest concentration

( $10^{-6}$  M). Oligomerization of monomeric  $ind^+ - 1$ , compared with wild-type, repressor therefore appeared to be far less extensive.

The kinetics of repressor cleavage by the *recA* protease was then determined in a purified system by measuring one of the cleavage products. Aliquots were taken at various times and analysed on SDS-polyacrylamide gels; repressor concentration varied in twofold steps. The rates of cleavage were estimated by densitometric scanning of autoradiograms (Fig. 2) and are summarized in Table 1. In the experiment shown, excess unlabelled  $ind^+$  repressor, added to a low concentration of labelled  $ind^+$  repressor, partially protected the latter from cleavage by *recA* protein. Furthermore, as repressor concentration increased, its rate of cleavage progressively decreased from that expected if it were proportional to repressor concentration. Consistent with previous studies measuring inactivation of repressor and as argued previously<sup>3</sup> (see above), this effect was not due to saturation of the protease, because (1) the same pattern was observed at a higher protease concentration (Table 1) and (2) protease monomers were present in at least a sixfold molar excess over repressor monomers.

By contrast,  $ind^+ - 1$  repressor was cleaved at a rate nearly proportional to repressor concentration, deviating only slightly at the highest concentration tested ( $1.4 \times 10^{-6}$  M) (compare Fig. 2a and b; Table 1). As both wild-type and mutant repressors were cleaved at about the same rate ( $4 \times 10^{-9}$  M; Table 1) and both are largely monomers at this concentration (refs 9, 10 and Fig. 1), we conclude that monomers of the two repressors have roughly equal sensitivity to protease. We propose that the  $ind^+ - 1$  repressor is more sensitive at high concentrations because of its reduced ability to form dimers. These findings therefore provide further evidence that repressor monomers are a preferred substrate for *recA* protease<sup>3</sup>.

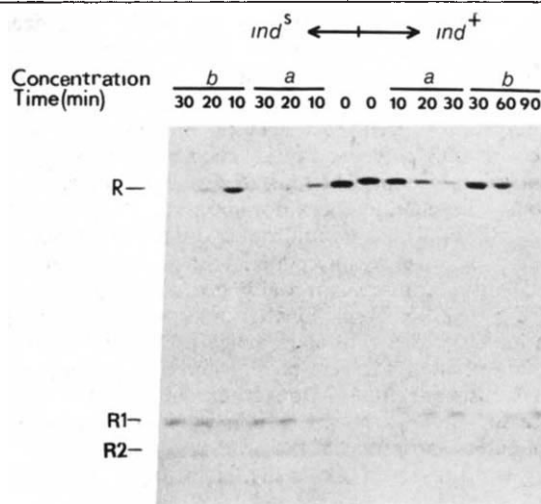
The above properties of  $ind^+ - 1$  repressor can probably account for the behaviour of the  $\lambda ind^+ - 1$  mutant *in vivo*. At  $10^{-7}$  M, the concentration estimated to be present in lysogenic cells<sup>9</sup>, a greater relative amount of  $ind^+ - 1$  repressor should be present as monomers, susceptible to the *recA* protease. Consequently, in a weakly induced cell, in which only a small amount of protease should be activated, a relatively high rate of repressor cleavage would be expected, leading to efficient induction. On the other hand, in a lysogen of wild-type  $\lambda$ , more repressor should be in the form of dimers resistant to cleavage, and should, therefore, bind to operators and maintain the repressed state.

We acknowledge the gift of  $\lambda precA$  (*tif-1*) c1857 Q73 S7 from R. Yocum and the communication of unpublished observations

**Table 1** Influence of repressor concentration on cleavage rate by the *recA* protease

| <i>recA</i> concentration ( $\mu g ml^{-1}$ ) | Repressor concentration (nM) | Rate of repressor cleavage (pmol $ml^{-1} min^{-1}$ ) of |                  |
|-----------------------------------------------|------------------------------|----------------------------------------------------------|------------------|
|                                               |                              | <i>ind^+</i>                                             | <i>ind^+ - 1</i> |
| 70                                            | 4                            | 0.10, 0.15                                               | 0.18, 0.20       |
|                                               | 35                           | 0.8, 0.9                                                 | 1.6, 1.7         |
|                                               | 140                          | 2.0                                                      | —                |
|                                               | 1,400                        | 11, 8                                                    | 42, 46           |
| 300                                           | 35                           | 2.0                                                      | —                |
|                                               | 140                          | 6                                                        | —                |
|                                               | 1,400                        | 24                                                       | —                |

$^{35}S$ -labelled repressor was diluted with buffer or unlabelled repressor, allowed to reach monomer-dimer equilibrium and then incubated with *recA* protein as described in Fig. 2 legend. At 2.5-min intervals, samples were removed until the reaction had run to completion. The reaction was stopped by adding samples to stopping buffer as described<sup>17</sup> and the samples subjected to polyacrylamide gel electrophoresis. Films exposed to the gels were scanned by a densitometer (Quick Scan R&D, Helena Laboratories) to determine the rate of appearance of label in the upper repressor fragment (R1). The time at which 50% of the maximal level of cleavage had occurred was determined from a plot of film optical density at the position of the R1 fragment versus time of incubation. The rate of repressor cleavage in pmol per ml per min was calculated by multiplying the average fractional rate of repressor cleavage during this time by initial repressor concentration. Values separated by commas are measurements from independent experiments. In the reactions in which *recA* concentrations were 300  $\mu g ml^{-1}$ , DNA was 25  $\mu g ml^{-1}$ . Varying the ratio of unlabelled to labelled repressor in the reaction mixtures did not influence the results.



**Fig. 2** Influence of repressor concentration on cleavage by *recA* protein. <sup>35</sup>S-labelled repressor was diluted to  $3.5 \times 10^{-8}$  M in tubes containing 12 mM Tris pH 7.5, 2 mM MgCl<sub>2</sub>, 0.43 mM CaCl<sub>2</sub>, 20 mM NaCl, 43 mM KCl, 0.54 mM EDTA, 1 mM potassium phosphate pH 7.5, 2.4 mM dithiothreitol, 9% glycerol, 165 g ml<sup>-1</sup> bovine serum albumin, 1 mM  $\gamma$ -thio-ATP and either with (b) or without (a) unlabelled repressor at  $1.4 \times 10^{-6}$  M. Following incubation for 90 min at 0 °C to establish monomer-dimer equilibrium, denatured DNA (6  $\mu$ g ml<sup>-1</sup>) was added, the tubes incubated for 5 min at 37 °C, *recA* (*tif* mutant) protein added to 70  $\mu$ g ml<sup>-1</sup> and incubation continued at 37 °C. Samples (30  $\mu$ l) were taken at the indicated time and loaded on a 15–25% SDS polyacrylamide gradient gel and electrophoresed. Gels were stained with bromophenol blue, photographed, prepared for fluorography and subjected to autoradiography. R marks the position of the repressor monomer band, R1 and R2 the positions of the carboxyl- and amino-terminal fragments of repressor respectively. *ind*<sup>s</sup>-1 repressor results are shown on the left half of the gel, *ind*<sup>+</sup> on the right; the centre lanes show uncleaved repressor. Times indicate time of sample removal. Note the later sample times for *ind*<sup>+</sup> repressor cleavage at the high repressor concentration and the increased mobility of *ind*<sup>s</sup>-1 repressor and its carboxyl-terminal fragment (R1) compared with *ind*<sup>+</sup> repressor. Repressor bands on stained gels showed intensity changes similar to those on the autoradiogram (not shown). Similar results were also obtained when wild-type *recA* protein was substituted for the *tif* mutant *recA* protein. *recA* protein was purified from extracts of DM1590 *sup*<sup>+</sup> *spr*-51 *lexA3* *tif*-1 *sfiA11* infected with  $\lambda$  *precA* (*tif*-1) cI857 Q73 S7 (prepared by R. Yocum and genotype confirmed by us) at a multiplicity of infection of 3. After incubation for 2 h at 37 °C, each litre of cells was collected by centrifugation, suspended in 2 ml 20 mM Tris pH 7.5,  $5 \times 10^{-4}$  M EDTA,  $10^{-3}$  M dithiothreitol, 10% w/v sucrose, 0.2 M NaCl and stored frozen at -20 °C. After thawing, cells were disrupted by sonication and centrifuged for 30 min at 24,000g. *recA* protein was then purified by polymin P and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation, followed by chromatography on phosphocellulose in potassium phosphate buffer and finally by sedimentation on sucrose gradients as described elsewhere<sup>16</sup>. The protein was 99% pure as judged by gel electrophoresis. One A<sub>280</sub> unit of *recA* protein is equivalent to 0.59 mg ml<sup>-1</sup> (J. Roberts, personal communication).

## *recA*-independent general genetic recombination of plasmids

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The small circular genomes of several bacteriophages and a number of plasmids have been used extensively to study various aspects of the mechanism of genetic recombination. Several studies have demonstrated that the interconversion between the circular monomers and larger circular oligomers of many plasmid DNAs is mediated, at least in part, by the pathway which carries out genetic recombination in wild-type *Escherichia coli*<sup>1–4</sup>. The *recA*, *recB* and *recC* mutations block conjugation-mediated recombination in *E. coli*<sup>5,6</sup>. However, Clark and his co-workers<sup>7,8</sup> isolated two mutations, *sbcA* and *sbcB*, which indirectly suppress the recombination deficiency of *recB* and *recC* mutations. These indirect suppressor mutations seem to cause recombination by other pathways which require the *recA* gene product as well as products of other genes such as *recF*, *recJ* and *recM*, which are not normally required for recombination in *E. coli*<sup>9,10</sup>. Here we report an investigation of the effect of recombination-deficient mutations on plasmid recombination in *E. coli*. We show that *sbcA* mutations induce a new plasmid recombination pathway that is 10–15 times more efficient than the normal wild-type recombination pathway and independent of the *recA* and *recF* functions.

The *E. coli* plasmid pVH51 is a 3.8-kilobase (kb) plasmid which codes for immunity to colicin E1 (ref. 11). It forms small numbers of circular oligomers by recombination in wild-type *E. coli* strains apparently because it lacks a genetic element required for high rates of recombination<sup>4,12</sup>. To study the effect of various recombination-deficient mutations on the recombination of pVH51 DNA, monomeric pVH51 DNA was purified by electrophoresis on preparative agarose gels and used to transform *E. coli* AB1157 and a number of isogenic strains containing different recombination-deficient mutations. Plasmid DNA was then purified from the resulting strains and examined for the presence of oligomeric forms by electrophoresis on agarose gels. The results (Fig. 1a) showed that pVH51 DNA formed very few circular oligomers when maintained in *E. coli* AB1157 (2–4% by weight, determined by electron microscopy) or other wild-type *E. coli* strains (results not shown). When pVH51 DNA was purified from *recA* strains (Fig. 1b), *recB recC* strains (Fig. 1c), *recF* strains (Fig. 1d) and *recB recC recF* strains (Fig. 1e) low numbers of circular oligomers were found (<1% by weight). These mutations reduced the numbers of oligomers but their effect is not pronounced as pVH51 DNA forms few oligomers in wild-type *E. coli*. When strains containing *recB recC sbcA* mutations (Fig. 1f), *recB recC sbcA recF* mutations (Fig. 1g) and *recB recC sbcA recA* mutations (Fig. 1h) were similarly examined, the pVH51 DNA was found to contain high levels of circular oligomers. These results show that the *sbcA* mutation stimulated the recombination of pVH51 DNA and that the reaction did not require the *recA* and *recF* functions.

Studies on the structure of the oligomeric forms of bacteriophage and plasmid DNAs have identified a novel oligomeric form which looks like a figure-8 (refs 13–17) and has many of the properties of a recombination intermediate postulated by Holliday<sup>18</sup> and others<sup>19</sup> to explain both gene conversion and reciprocal exchanges. Similar DNA molecules have been

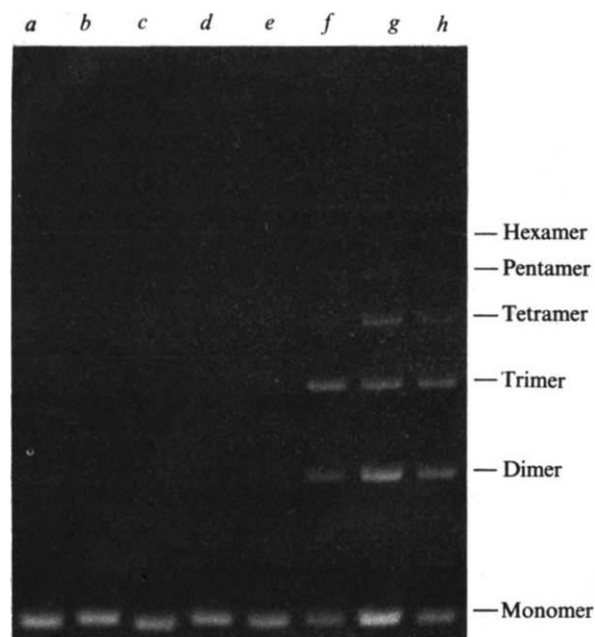
by R. Yocum, R. Sauer and J. Roberts. We also thank S. Edmiston and I. Sassoon for technical and secretarial assistance respectively. This research was supported by NIH grant GM24496 and NSF grant PCM-7912059.

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- Roberts, J. W., Roberts, C. W. & Craig, N. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4714–4718 (1978).
- Horiuchi, T. & Inokuchi, H. *J. molec. Biol.* **23**, 217–224 (1967).
- Phizicky, E. M. & Roberts, J. W. *J. molec. Biol.* **139**, 319–328 (1980).
- Pabo, C. O., Sauer, R. T., Sturtevant, J. M. & Ptashne, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1608–1612 (1979).
- Sauer, R. T., Pabo, C. O., Meyer, B. J., Ptashne, M. & Backman, K. C. *Nature* **279**, 396–400 (1979).
- Roberts, J. W. & Roberts, C. W. *Proc. natn. Acad. Sci. U.S.A.* **72**, 147–151 (1975).
- Bailone, A., Levine, A. & Devoret, R. *J. molec. Biol.* **131**, 553–572 (1979).
- Blanco, M. & Pomes, L. *Molec. gen. Genet.* **154**, 287–292 (1977).
- Pirrotta, V., Chadwick, P. & Ptashne, M. *Nature* **227**, 41–44 (1970).
- Sauer, R. T. thesis, Harvard Univ. (1979).
- Smith, G. R. *Virology* **64**, 544–552 (1975).
- Mount, D. W. *Molec. gen. Genet.* **145**, 165–167 (1976).
- Backman, K. & Ptashne, M. *Cell* **13**, 65–71 (1978).
- Muller-Hill, B. *Prog. Biophys. molec. Biol.* **30**, 227–252 (1975).
- Johnson, A. D., Pabo, C. O. & Sauer, R. T. *Meth. Enzym.* **65**, 839–855 (1980).
- Craig, N. L. & Roberts, J. W. *Nature* **283**, 26–30 (1980).
- Little, J. W., Edmiston, S. H., Pacelli, L. Z. & Mount, D. W. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3225–3229 (1980).

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**Fig. 1** Electrophoretic analysis of pVH51 DNA purified from isogenic *E. coli* strains containing recombination-deficient mutations. Monomeric pVH51 DNA was purified from *E. coli* RK1058 *recA1* (pVH51) as described elsewhere<sup>37</sup> and the monomeric DNA was further purified by electrophoresis on preparative agarose slab gels<sup>38</sup>. The DNA was then used to transform the *E. coli* strain of interest to resistance to colicin E1<sup>39</sup>. Twelve 10-ml cultures of L-broth were inoculated with a single transformant, grown to a density of  $4 \times 10^8$  cells ml<sup>-1</sup> at 37°C and chloramphenicol was added to 250 µg ml<sup>-1</sup>. After shaking for 16 h, plasmid DNA was purified from the cultures and analysed by electrophoresis on a 0.5% agarose slab gel<sup>40</sup>. One representative DNA sample from each strain was further purified by centrifugation to equilibrium in CsCl-Et density gradients<sup>37</sup> and analysed by electrophoresis on a 0.5% agarose slab gel. The DNA samples shown were purified from: a, AB1157 wild type; b, JC2924 *recA56*; c, JC5519 *recB21 recC22*; d, JC9239 *recF143*; e, JC3881 *recB21 recC22 recF143*; f, JC8679 *recB21 recC22 sbcA23*; g, JC9610 *recB21 recC22 recF143 sbcA23*; and h, JC9604 *recA56 recB21 recC22 sbcA23*.

observed in many systems used to study genetic recombination<sup>20-23</sup>. A recombination model for circular genomes which integrates all these observations has been discussed in detail elsewhere<sup>24</sup>. Electrophoretic analysis cannot easily distinguish between circular oligomers, catenated oligomers and figure-8 molecules. Therefore pVH51 DNA purified from a *recB recC sbcA recA* strain was examined by electron microscopy. The results (Fig. 2 and Table 1) demonstrated that >95% of the oligomers observed in Fig. 1 were circular oligomers. The level of circular oligomers in *recB recC sbcA recA* strains was ~20-fold higher than in wild-type strains and 100-fold higher than in *recA* strains. Low levels of catenated oligomers (Fig. 2e) and figure-8 molecules (Fig. 2f) were also observed. To quantitate accurately the levels of figure-8 molecules present, the pVH51 DNA was converted to a mixture of X-forms (Fig. 2g, h) and linear monomers by digestion with *KpnI* (one cleavage site

per monomer<sup>25</sup>) in conditions that prevent branch migration<sup>15,24</sup> before mounting the DNA for electron microscopy. The results of this analysis (Table 1) verified the presence of figure-8 molecules in the DNA preparation and suggested that figure-8 recombination intermediates might function in the recombination reaction observed in *recB recC sbcA recA* strains.

The exact relationship between plasmid recombination and the interconversion of plasmid oligomers is unknown; thus we sought to develop a procedure that would allow a more direct measure of plasmid recombination. Tetracycline-sensitive (*tet*<sup>-</sup>) mutations of the ampicillin-resistant, tetracycline-resistant plasmid pBR322 (ref. 26) were isolated by digesting pBR322 DNA with either *Bam*HI or *Sal*I, filling in the resulting 'sticky ends' with reverse transcriptase, followed by cyclization in blunt end-ligation conditions. The resulting plasmids contained 4-base pair (bp) insertions, inactivating the tetracycline-resistance gene and either the *Bam*HI (pRDK2) or *Sal*I (pRDK3) cleavage site. These *tet*<sup>-</sup> mutations had reversion frequencies of  $<10^{-9}$ . The two plasmid DNAs were then digested to unit-length linear DNA molecules using *Eco*RI and joined to each other with T4 DNA ligase to construct a circular dimer containing one copy of each of the two different mutant alleles in tandem repeat (pRDK301). When this plasmid DNA is transformed into a recombination-proficient *E. coli* strain, it should recombine to yield a functional tetracycline-resistance gene at a characteristic frequency and the production of *tet*<sup>r</sup> progeny during the growth of the initial *tet*<sup>-</sup> transformant should be related to the recombination frequency. Control experiments (data not shown) demonstrated that recombination occurred to produce *tet*<sup>r</sup> progeny and that it resulted in the rearrangement of the inactivated restriction endonuclease cleavage sites with respect to each other. The construction of these plasmids and their use in a fluctuation test<sup>27</sup> to determine the frequency of plasmid recombination will be described in detail elsewhere. A similar assay has been used to study SV40 recombination<sup>28</sup>.

To determine the effect of recombination-deficient mutations on plasmid recombination, a set of isogenic *E. coli* strains containing different recombination-deficient mutations was transformed to *amp*<sup>r</sup> with pRDK301 DNA and the production of *tet*<sup>r</sup> progeny measured as described in Table 2. The effect of fluctuation<sup>27</sup> on the measurements was mitigated by making a number of independent determinations and averaging the results. The results (Table 2) showed that recombination to *tet*<sup>r</sup> in wild-type backgrounds (*sbcA*<sup>+</sup>) was reduced by *recA* and *recF* mutations but was stimulated by *recB recC* mutations. In contrast, recombination in *recB recC sbcA* strains was stimulated by *recA* and *recF* mutations. The difference in the effect of *recA* and *recF* mutations on recombination in wild-type and *recB recC sbcA* strains was quite striking—recombination in *recB recC sbcA recA* strains was 11,700 times higher than in *recA* strains and 500 times higher in *recB recC sbcA recF* strains than in *recF* strains. This effect required the *sbcA* mutation as the *recA* and *recF* mutations decreased recombination in *recB recC* strains. Furthermore, the suppression of *recA* mutations by *sbcA* mutations occurred in the presence of functional *recB* and *recC* genes.

These results demonstrate that plasmid recombination in wild-type *E. coli* requires the *recA* and *recF* gene products, which is consistent with the findings of others<sup>17,29</sup>. Note that the effect of *recF* mutations on plasmid recombination differs

**Table 1** Analysis of pVH51 DNA isolated from JC9604 *recA56 recB21 recC22 sbcA23*

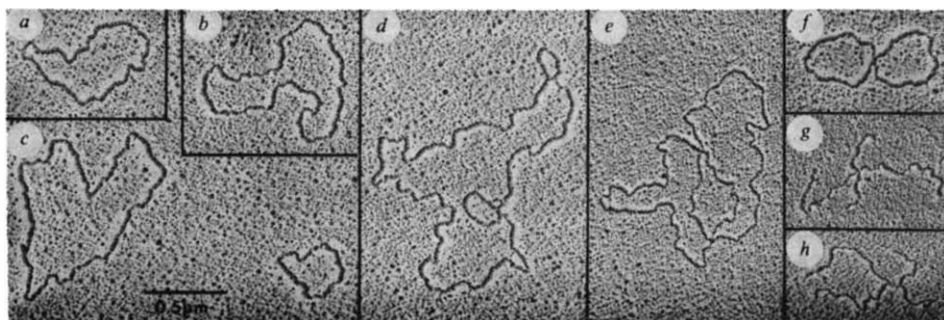
|                      | Distribution of oligomeric forms |       |       |                   |       |       |        | Catenaes<br>All types | Figure-8s<br>All types  |
|----------------------|----------------------------------|-------|-------|-------------------|-------|-------|--------|-----------------------|-------------------------|
|                      | 1-mer                            | 2-mer | 3-mer | Circular<br>4-mer | 5-mer | 6-mer | >6-mer |                       |                         |
| No. (%) <sup>*</sup> | 71.5                             | 16.1  | 7.0   | 3.5               | 0.4   | 0.4   | 0.1    | 0.7                   | 0.2 (0.14) <sup>†</sup> |
| Weight (%)           | 48.2                             | 21.7  | 14.1  | 9.4               | 1.4   | 1.7   | 0.8    | 1.9                   | 0.7                     |

1-mer, monomer; 2-mer, dimer; 3-mer, trimer, etc.

<sup>\*</sup> Based on the scoring and measurement of 716 molecules, equivalent to 1,061 monomer units.

<sup>†</sup> Based on the scoring of 9,995 linear monomers and 10 X-forms.

**Fig. 2** Electron microscopic analysis of pVH51 DNA purified from JC9604 *recA56 recB21 recC22 sbcA23*. The plasmid DNA preparation analysed in Fig. 1h was treated with  $\gamma$ -irradiation to relax the super-twisted DNA molecules or digested with *KpnI* for 10 min at 10 °C (in conditions supplied by New England Biolabs). After each treatment the DNA was mounted for electron microscopy by the aqueous technique essentially as described elsewhere<sup>13,37</sup>. The representative DNA molecules shown are: a, circular dimer; b, circular trimer; c, circular monomer and circular tetramer; d, circular pentamer; e, catenane of two trimers; f, figure-8 containing two monomer units; and g, h, X-forms produced by *KpnI* digestion.



significantly from that on conjugation-mediated recombination<sup>9,10</sup>. The effect of *recB recC* mutations is complex as they stimulated genetic recombination of pBR322 but not oligomerization of pVH51. These observations are not inconsistent and are due to the use of two different assays to measure recombination, as we have recently shown that the products of recombination in *recB recC* strains are almost exclusively circular monomers. These results suggest that the *recB recC* gene product (exonuclease V) is probably involved in the resolution of recombination intermediates<sup>29</sup>. We have also shown that *E. coli* strains containing mutations in the gene for topoisomerase I are deficient in plasmid recombination (R.A.F., R.K. and J. C. Wang, unpublished results).

Our results have demonstrated that *sbcA* mutations induce a recombination pathway that will catalyse plasmid recombination more efficiently than the normal wild-type recombination pathway. Interestingly, this pathway does not require functional *recA* and *recF* genes, which suggests that the mechanism of recombination catalysed by it differs considerably from wild-type recombination. Similar results, obtained in studies which examined the effect of *sbcA* mutations on recombination of bacteriophage  $\lambda$  *red*<sup>-</sup>, suggest that plasmid and  $\lambda$  recombination may be similar in strains containing *sbcA* mutations<sup>30</sup>. Presumably, plasmid recombination in *recB recC sbcA* strains requires proteins coded for by the *Rac* cryptic prophage as well as other unidentified *E. coli* gene products<sup>31</sup>. We have not tested the effect of *sbcB* mutations on plasmid recombination as these mutations affect the ability of *E. coli* to maintain plasmids<sup>32</sup>.

Consideration of the data available shows that the recombination-deficient mutations have different effects depending on the type of *E. coli* recombination event analysed<sup>6,9,10,17,29,33,34</sup>. These observations suggest that the different homology-dependent recombination events differ structurally and there-

fore require different proteins to catalyse them. It also seems likely that proteins like exonuclease V (the product of the *recB* and *recC* gene<sup>35,36</sup>) may function differently in different recombination events. This could explain why *recB recC* mutations affect the formation of stable recombinants in topologically similar  $\lambda \times F'$  and plasmid crosses differently<sup>33</sup>. The nature of the significant differences between all these recombination events is not fully understood.

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- Hobom, G. & Hogness, D. S. *J. molec. Biol.* **88**, 65-87 (1974).
- Bedbrook, J. & Ausubel, F. *Cell* **9**, 707-716 (1976).
- Potter, H. & Dressler, D. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4168-4172 (1977).
- Kolodner, R., Joseph, J., James, A., Dean, F. & Doherty, M. J. *ICN-UCLA Symp. molec. cell. Biol.* **19**, 933-939 (1980).
- Clark, A. J. & Margulies, A. D. *Proc. natn. Acad. Sci. U.S.A.* **53**, 451-459 (1965).
- Emmerson, P. T. & Howard-Flanders, P. *J. Bact.* **93**, 1729-1731 (1967).
- Barbour, S. D., Nagaishi, H., Templin, A. & Clark, A. J. *Proc. natn. Acad. Sci. U.S.A.* **67**, 128-135 (1970).
- Kushner, S. R., Nagaishi, H. & Clark, A. J. *Proc. natn. Acad. Sci. U.S.A.* **68**, 824-827 (1971).
- Horii, Z.-I. & Clark, A. J. *J. molec. Biol.* **80**, 327-344 (1973).
- Clark, A. J. *ICN-UCLA Symp. molec. cell. Biol.* **19**, 891-899 (1980).
- Hershfield, V., Boyer, H. W., Chow, L. & Helinski, D. R. *J. Bact.* **126**, 447-453 (1976).
- James, A. A., Morrison, P. M. & Kolodner, R. *Proc. natn. Acad. Sci. U.S.A.* (submitted).
- Gordon, C. N., Rush, M. G. & Warner, R. C. *J. molec. Biol.* **47**, 495-503 (1970).
- Thompson, B. J. *et al. J. molec. Biol.* **91**, 409-419 (1975).
- Thompson, B. J., Camien, M. N. & Warner, R. C. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2299-2303 (1976).
- Potter, H. & Dressler, D. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3000-3004 (1976).
- Potter, H. & Dressler, D. *Cold Spring Harb. Symp. quant. Biol.* **43**, 969-989 (1978).
- Holliday, R. *Genet. Res.* **5**, 282-304 (1964).
- Meselson, M. & Radding, C. M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 358-361 (1975).
- Broker, T. R. *J. molec. Biol.* **81**, 1-16 (1973).
- Tsujimoto, Y. & Ogawa, A. *J. molec. Biol.* **106**, 423-436 (1976).
- Bell, L. & Byers, B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3445-3449 (1979).
- Wolgemuth, D. J. & Hsu, M.-T. *Nature* **287**, 168-171 (1980).
- Warner, R. C., Fishel, R. A. & Wheeler, F. C. *Cold Spring Harb. Symp. quant. Biol.* **43**, 957-968 (1978).
- Hardies, S. C. *et al. J. biol. Chem.* **254**, 5527-5534 (1979).
- Bolivar, F., Rodriguez, R. L., Betlach, M. C. & Boyer, H. W. *Gene* **2**, 75-93 (1977).
- Luria, S. E. & Delbruck, M. *Genetics* **28**, 491-504 (1943).
- Wake, C. T. & Wilson, J. H. *Cell* **21**, 141-148 (1980).
- James, A. A., Morrison, P. M. & Kolodner, R. *J. molec. Biol.* (submitted).
- Gillen, J. R. & Clark, A. J. in *Mechanisms of Genetic Recombination* (ed. Grell, R. F.) 123-136 (Plenum, New York, 1974).
- Gillen, J. R., Willis, D. K. & Clark, A. J. *J. Bact.* **145**, 521-532 (1981).
- Vapnek, D., Alton, N. K., Bassett, C. L. & Kushner, S. R. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3492-3496 (1976).
- Porter, R. D., McLaughlin, T. & Low, B. *Cold Spring Harb. Symp. quant. Biol.* **43**, 1043-1047.
- Stahl, F. W. *A. Rev. Genet.* **13**, 7-24 (1979).
- Barbour, S. D. & Clark, A. J. *Proc. natn. Acad. Sci. U.S.A.* **65**, 955-961 (1970).
- Goldmark, P. J. & Linn, S. *Proc. natn. Acad. Sci. U.S.A.* **67**, 434-438 (1972).
- Kolodner, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4847-4851 (1980).
- Thuring, R. W. J., Sanders, J. P. M. & Borst, P. *Analyt. Biochem.* **66**, 213-220 (1975).
- Wensink, P. C., Finnegan, D. J., Donelson, D. J. & Hogness, D. S. *Cell* **3**, 315-325 (1974).
- Klein, R. D., Selsing, E. & Wells, R. D. *Plasmid* **3**, 88-91 (1980).

**Table 2** Effect of recombination-deficient mutations on recombination of pRDK301

| Genotype tested |             |             |             | Proportion of <i>tet</i> <sup>r</sup> progeny |                      |
|-----------------|-------------|-------------|-------------|-----------------------------------------------|----------------------|
| <i>recA</i>     | <i>recB</i> | <i>recC</i> | <i>recF</i> | <i>sbcA</i> <sup>+</sup>                      | <i>sbcA23</i>        |
| +               | +           | +           | +           | $6.4 \times 10^{-4}$                          | ND                   |
| 56              | +           | +           | +           | $8.1 \times 10^{-7}$                          | $5.5 \times 10^{-4}$ |
| +               | +           | +           | 143         | $1.6 \times 10^{-5}$                          | ND                   |
| +               | 21          | 22          | +           | $2.9 \times 10^{-3}$                          | $3.1 \times 10^{-3}$ |
| 56              | 21          | 22          | +           | $2.1 \times 10^{-5}$                          | $9.5 \times 10^{-3}$ |
| +               | 21          | 22          | 143         | $4.3 \times 10^{-4}$                          | $7.9 \times 10^{-3}$ |

The *E. coli* strain of interest was transformed to ampicillin resistance with pRDK301 DNA<sup>39</sup>. A 10-ml culture of L-broth containing 50  $\mu$ g ml<sup>-1</sup> ampicillin was inoculated with an entire transformant colony and incubated at 37 °C with shaking until a cell density of  $5 \times 10^7$  was obtained. Samples were then plated out on L-broth plates containing either 50  $\mu$ g ml<sup>-1</sup> ampicillin or 50  $\mu$ g ml<sup>-1</sup> ampicillin + 25  $\mu$ g ml<sup>-1</sup> tetracycline to determine the proportion of Tet<sup>r</sup> cells present in the culture. The values shown are the average results obtained from an analysis of 10 independent cultures. The strains tested are those described in the Fig. 1 legend, and RK1064 *recA56 recB21 recC22* and RK1289 *recA56 sbcA23*. All are isogenic with *E. coli* AB1157. ND, not determined.



## *In vivo* decoding rules in *Schizosaccharomyces pombe* are at variance with *in vitro* data

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On the basis of *in vitro* experiments, conflicting hypotheses have been proposed for the assignment of tRNA isoacceptors to the reading of synonymous codons<sup>1,2</sup>. Here we present a genetic approach for studying the decoding capacity of serine isoacceptors *in vivo*. Cells of *Schizosaccharomyces pombe* have been depleted of tRNA<sup>Ser</sup><sub>UCA</sub> by mutating the two genes known to code for this isoacceptor, *sup3* and *sup9*, first to UGA nonsense suppressor alleles and then to suppressor inactive alleles carrying secondary lesions. The lethality resulting from the combination of inactive alleles at both loci indicates that tRNA<sup>Ser</sup><sub>UCA</sub> is essential for growth. Disregarding the remote possibility that this tRNA has vital roles other than in protein synthesis, we conclude that its function in translating UCA cannot be taken over by any other serine tRNA, including the major inosine-containing isoacceptor. This is at variance with both the wobble rule for inosine proposed by Crick<sup>1</sup> and the 'two out of three' reading scheme proposed by Lagerkvist<sup>2</sup>, and suggests that decoding rules deduced from *in vitro* data do not necessarily apply to the *in vivo* situation.

The wobble hypothesis of Crick states that some bases at the first anticodon position can pair with the third codon base in a non-standard way, allowing UG, GU, IU, IC and IA pairs beside the standard ones<sup>1</sup>. Experimental support for the wobble rules is provided by triplet binding assays with *Escherichia coli* ribosomes<sup>3</sup>. However, exceptions to this rule have been described: extended wobbling is observed in mitochondria where U recognizes codons ending with U, C, A and G (refs 4–6). The same seems to be true for a tRNA<sup>Gly</sup> in *Mycoplasma mycoides*<sup>7</sup>. On the other hand, some modifications of U in the wobble position have been proposed<sup>8</sup> to restrict the recognition to codons ending with A.

A further discrepancy (the subject of the present study) concerns the wobble rule for inosine. In the eukaryotes studied so far a major tRNA<sup>Ser</sup> with the anticodon IGA was found and thought to be responsible for the recognition of the codons UCU, UCC and UCA<sup>9,10</sup>. However, in *Saccharomyces cerevisiae*<sup>11</sup> and *Schizosaccharomyces pombe*<sup>12</sup> a UCA-reading tRNA<sup>Ser</sup> with the anticodon U\*GA has also been identified. We present here genetic evidence which suggests that in *S. pombe*, this minor isoacceptor evolved because the major serine tRNA is unable to translate UCA *in vivo*.

Our approach was to deplete cells of a specific active tRNA by genetic means and to observe the consequences on cell growth. The genes *sup3*<sup>+</sup> and *sup9*<sup>+</sup> in wild-type strains of *S. pombe* code for the tRNA<sup>Ser</sup> isoacceptor having the anticodon U\*GA. This is inferred from genetic evidence<sup>12</sup> and from the nucleotide sequence of the UGA-reading *sup3-e* opal suppressor tRNA<sup>Ser</sup> (*e* for efficient; anticodon U\*CA, where U\* is partly 2-thiouridine and partly 5-(methoxycarbonylmethyl)-uridine)<sup>13</sup>. A corresponding situation holds for the tRNA<sup>Leu</sup> isoacceptor (anticodon U\*AA) which is coded for by the genes *sup8*<sup>+</sup> and *sup10*<sup>+</sup>. Mutations at the anticodon site of these two genes lead to efficient opal suppressor alleles and the product of one of these (*sup8-e* tRNA) has been sequenced<sup>14</sup>. Strains carrying such opal suppressors thus produce either a tRNA<sup>Ser</sup> or a tRNA<sup>Leu</sup> whose anticodon U\*CA no longer recognizes UCA or UUA, respectively. All four opal suppressors have been inactivated by secondary mutations that map within the respective suppressor genes at different sites<sup>15</sup>. These secondary lesions are likely to abolish either the biosynthesis or function of the suppressor tRNAs.

By crossing strains carrying inactive suppressor alleles at different loci, recombinants harbouring the mutant alleles at both loci can be obtained (Table 1). Three types of tetrads are observed in such crosses and they occur in frequencies expected for a two-factor cross of randomly assorting genes: 1/6 parental ditype tetrads (PD), 2/3 tetratype tetrads (T) and 1/6 non-parental ditype tetrads (NPD). The four suppressor genes studied are not linked genetically to each other. The PDs consist of four colonies. In tetratypes and NPDs, respectively, one and two recombinant spores that carry two inactivated suppressor genes coding for the same tRNA (that is, *sup3-e*, *rX sup9-e*, *rX* or *sup8-e*, *rX sup10-e*, *rX*; *r* for reversion) fail to form colonies on yeast extract agar. In fact they are unable to undergo the first cell division. Whereas tRNA<sup>Ser</sup>-deficient spores elongate to form structures resembling a premitotic stage, tRNA<sup>Leu</sup>-deficient spores do not germinate at all. Identical results were obtained when the spores were placed on minimal agar supplemented with adenine (the only requirement of the strains involved) and incubated at 25°C as well as at standard 30°C (data not shown). Even when one or both partners of a cross carry an active suppressor gene (no inactivating second-site mutation present in one or both suppressor genes of a given pair), recombinants lacking wild-type alleles fail to grow. However, strains carrying two active suppressors inserting different amino acids (for example, *sup3-e sup8-e*) are viable<sup>16</sup>. Thus the lethality observed in combinations of two mutated tRNA<sup>Ser</sup> genes and two mutated tRNA<sup>Leu</sup> genes, respectively, is due to the loss of the corresponding wild-type tRNAs and not due to excessive suppression.

The indispensable nature of tRNA<sup>Ser</sup><sub>UCA</sub> and tRNA<sup>Leu</sup><sub>UUA</sub> demonstrated by these results is interpreted to mean that in *S. pombe*, these are the only tRNAs able to read UCA and UUA, respectively with an efficiency sufficient for cell growth. The alternative explanation which assumes an essential function of the specific tRNA<sup>Ser</sup><sub>UCA</sub> and tRNA<sup>Leu</sup><sub>UUA</sub> isoacceptors in processes unrelated to protein synthesis cannot be excluded at present, but seems unlikely.

As our data indicate that in *S. pombe* the codon UCA is read efficiently only by the tRNA<sup>Ser</sup> with the anticodon U\*GA (a minor isoacceptor), we have purified the major tRNA<sup>Ser</sup> from this organism. We are now sequencing this tRNA and HPLC of tRNA digests<sup>17</sup> has revealed that it indeed contains inosine (our unpublished results). The lack of efficient UCA recognition by the inosine-carrying *S. pombe* serine tRNA is paralleled by the observation made in *S. cerevisiae* that no serine-inserting ochre suppressor tRNAs were found to be derived from the inosine-carrying isoacceptor<sup>11</sup>. We conclude from this that in both yeasts, wobbling of inosine when present in the first position of the anticodon IGA of the major tRNA<sup>Ser</sup> isoacceptor permits recognition of the serine UGX codons ending with U or C only.

The finding that the codon UCA is efficiently translated only by the serine tRNA containing the anticodon U\*GA also argues against decoding of the serine codon family UGX by the 'two out of three' method proposed by Lagerkvist<sup>2</sup> for those families of codons that differ only in the third position and code for a single amino acid. In this hypothesis, which is based on *in vitro* protein synthesis experiments, tRNAs pairing in a standard way at the first two codon positions are predicted to read all four codons of the family, irrespective of the nature of the wobble pair.

Similar data excluding decoding by the two out of three method have been reported for the UCG codon in *S. cerevisiae*, where it was demonstrated that tRNA<sup>Ser</sup> with the anticodon CGA is essential<sup>18,19</sup>. The same situation probably holds for *S. pombe*, as suggested by preliminary results obtained with a UGA suppressor derived from the gene coding for this tRNA (H. Amstutz, personal communication). In both yeasts the gene coding for the minor tRNA<sup>Ser</sup><sub>CGA</sub> has been sequenced<sup>11,20</sup>. Suppressors derived from it are haplo-lethal in both organisms. The essential nature of the tRNA<sup>Ser</sup> with the anticodon CGA which is demonstrated by this finding also shows that the hypermodified uridine at the wobble position of tRNA<sup>Ser</sup><sub>UCA</sub> prevents reading of the codon UCG. This agrees with earlier *in*

**Table 1** Analysis of pairwise crosses between *S. pombe* strains with inactivated serine tRNA and leucine tRNA genes

| Cross                     |                    | Tetrad types    |                                   |                                      |                                          |                        |
|---------------------------|--------------------|-----------------|-----------------------------------|--------------------------------------|------------------------------------------|------------------------|
|                           |                    | 4 colonies (PD) | 3 colonies and 1 lethal spore (T) | 2 colonies and 2 lethal spores (NPD) | Tetrads with superimposed growth defects | Total tetrads analysed |
| <b>tRNA<sup>Ser</sup></b> |                    |                 |                                   |                                      |                                          |                        |
| <i>sup3-e, r8</i> ×       | <i>sup9-e, r49</i> | 3               | 10                                | 1                                    | 2                                        | 16                     |
| <i>r15</i> ×              | <i>r101</i>        | 3               | 10                                | 1                                    | 2                                        | 16                     |
| <i>r30</i> ×              | <i>r104</i>        | 1               | 9                                 | 2                                    | 4                                        | 16                     |
| <i>r36</i> ×              | <i>r49</i>         | 1               | 12                                | 2                                    | 1                                        | 16                     |
| <i>r40</i> ×              | <i>r68</i>         | 2               | 10                                | 3                                    | 1                                        | 16                     |
| Total                     |                    | 10              | 51                                | 9                                    | 10                                       | 80                     |
| <b>tRNA<sup>Leu</sup></b> |                    |                 |                                   |                                      |                                          |                        |
| <i>sup8-e, r54</i> ×      | <i>sup10-e, r7</i> | 2               | 10                                | 2                                    | 0                                        | 14                     |
| <i>r54</i> ×              | <i>r17</i>         | 2               | 11                                | 0                                    | 1                                        | 14                     |
| <i>r54</i> ×              | <i>r24</i>         | 3               | 7                                 | 4                                    | 0                                        | 14                     |
| <i>r61</i> ×              | <i>r9</i>          | 5               | 8                                 | 0                                    | 1                                        | 14                     |
| <i>r139</i> ×             | <i>r15</i>         | 0               | 10                                | 3                                    | 1                                        | 14                     |
| <i>r169</i> ×             | <i>r17</i>         | 1               | 10                                | 3                                    | 0                                        | 14                     |
| <i>r440</i> ×             | <i>r24</i>         | 1               | 6                                 | 2                                    | 5                                        | 14                     |
| <i>r463</i> ×             | <i>r34</i>         | 3               | 9                                 | 0                                    | 2                                        | 14                     |
| Total                     |                    | 17              | 71                                | 14                                   | 10                                       | 112                    |

The genetic methods used are described elsewhere<sup>15</sup>. All strains involved carried the opal nonsense mutation *ade6-704* which causes an adenine requirement and the accumulation of a dark red pigment in cells grown on media containing limiting adenine concentrations (for example, yeast extract agar on which asci were dissected). Active opal suppressors (*sup3-e*, *sup9-e*, *sup8-e* and *sup10-e*) combined with *ade6-704* give prototrophic and white colonies, but the secondarily inactivated suppressors (*supX-e*, *rY*) have no effect on the *ade6-704* phenotype except in the following cases: *sup3-e, r8*, *-r36*, *sup8-e, r61*, *-r440*, *-r463*, *sup10-e, r17* and *-r24*. These strains display a pink rather than dark red pigment but are nevertheless auxotrophic. The segregations of colour differences in such cases are in complete agreement with the interpretation that the lethal spores contain mutant suppressor alleles at both loci involved. A minority of tetrads displayed in addition to the spore lethality caused by tRNA depletion, a superimposed lethality of unknown nature. The two types could be distinguished by microscopic examination, as non-growers due to tRNA defects had a constant, characteristic morphology.

*vitro* data<sup>8</sup> which indicate that certain modifications of U in the wobble position restrict a tRNA to the recognition of codons ending in A.

Further evidence against the hypothesis of two out of three reading has recently been presented by Murgola and Pagel<sup>21</sup>, who showed that in *E. coli* the glycine codon GGA is read *in vivo* only by tRNA<sup>Gly</sup> with the anticodon NCC, where N is a partially modified uridine<sup>22</sup>. The finding that in *S. pombe* the codon UUA is read efficiently only by the tRNA<sup>Leu</sup> with the anticodon U\*AA contrasts with earlier *in vitro* protein synthesis data obtained for yeast and *E. coli* tRNA which suggested that UUA may be read by more than one leucine isoacceptor<sup>23,24</sup>.

Clearly, *in vivo* decoding must be studied in more detail, but available evidence suggests that it does not simply conform to the hypotheses proposed on the basis of *in vitro* data.

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- Crick, F. H. C. *J. molec. Biol.* **19**, 548–555 (1966).
- Lagerkvist, U. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1759–1762 (1978).
- Söll, D., Cherayil, J. D. & Bock, R. M. *J. molec. Biol.* **29**, 97–112 (1967).
- Heckman, J. E., Sarnoff, J., Alzner-DeWeerd, B., Yin, S. & RajBhandary, U. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3159–3163 (1980).
- Bonitz, S. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 3167–3170 (1980).
- Barrell, B. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 3164–3166 (1980).
- Kilpatrick, M. W. & Walker, R. T. *Nucleic Acids Res.* **8**, 2783–2786 (1980).
- Agris, P. F., Söll, D. & Seno, T. *Biochemistry* **12**, 4331–4336 (1973).
- Zachau, H. G., Dütting, D. & Feldmann, H. *Hoppe-Seyler's Z. physiol. Chem.* **347**, 212–235 (1966).
- Ginsberg, T., Rogg, H. & Stachelin, M. *Eur. J. Biochem.* **21**, 249–257 (1971).
- Olson, M. V. *et al. Nature* **291**, 464–469 (1981).
- Kohli, J., Mao, J. & Söll, D. in *Genetics and Evolution of RNA Polymerase, tRNA and Ribosomes* (eds Osawa, S. *et al.*) 233–250 (University of Tokyo Press, 1980).
- Rafalski, A., Kohli, J., Agris, P. & Söll, D. *Nucleic Acids Res.* **6**, 2683–2695 (1979).
- Wetzel, R., Kohli, J., Altruda, F. & Söll, D. *Molec. gen. Genet.* **172**, 221–228 (1979).
- Hofer, F. *et al. Curr. Genet.* **1**, 45–61 (1979).
- Egel, R., Kohli, J., Thuriaux, P. & Wolf, K. A. *Rev. Genet.* **14**, 77–108 (1980).
- Davis, G. E., Gehrke, C. W., Kuo, K. C. & Agris, P. F. *J. Chromat.* **173**, 281–298 (1979).
- Piper, P. W. *J. molec. Biol.* **122**, 217–235 (1978).
- Etcheverry, T., Colby, D. & Guthrie, C. *Cell* **18**, 11–26 (1979).
- Mao, J., Schmidt, O. & Söll, D. *Cell* **21**, 509–516 (1980).
- Murgola, E. J. & Pagel, F. T. *J. molec. Biol.* **138**, 833–844 (1980).
- Roberts, J. W. & Carbon, J. J. *biol. Chem.* **250**, 5530–5541 (1975).
- Weissenbach, J., Dirheimer, G., Falcoff, R., Sanceau, J. & Falcoff, E. *FEBS Lett.* **82**, 71–76 (1977).
- Goldman, E., Holmes, W. M. & Hatfield, G. W. *J. molec. Biol.* **129**, 567–585 (1979).

## Wild-type tRNA<sup>Tyr</sup> reads the TMV RNA stop codon, but Q base-modified tRNA<sup>Tyr</sup> does not

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Although protein synthesis usually terminates when a stop codon is reached along the messenger RNA sequence, there are examples, mainly in viruses, of the stop codon being suppressed by a tRNA species. A strong candidate for this phenomenon occurs in tobacco mosaic virus (TMV) in the form of two proteins (110K and 160K, of molecular weights 110,000 and 160,000, respectively)<sup>1</sup>, sharing an N-terminus sequence, which are translated *in vitro* from a purified species of viral RNA. We have investigated the identity of the tRNA responsible for production of the 160K protein and show here that it is one of the tyrosine tRNAs. Another tyrosine tRNA, in which the first base of the anticodon is highly modified, does not act as a suppressor, indicating the possible regulatory function of such modifications.

As an assay system for readthrough, we have used simultaneous injection of TMV RNA and amber or ochre suppressor tRNAs from yeast into *Xenopus* oocytes; this results in synthesis of the 110K and 160K proteins<sup>2</sup>. In the process of screening RPC-5 fractionated *Drosophila melanogaster* tRNAs (21 suppressor stocks and Oregon wild type) for putative amber or ochre suppressors, we found that one fraction (*c*<sub>1</sub>) contained a strong readthrough activity in every RPC-5 profile tested, including the wild type (Fig. 1a, b). The fact that this readthrough activity is contained in every tRNA preparation indicated that we had discovered a natural suppressor tRNA for the TMV RNA 110K protein stop codon. Aminoacylation shows that this RPC-5 fraction contains a tRNA<sup>Tyr</sup> isoacceptor (Fig. 1a), a good candidate for such a suppressor. Purification of this tRNA to homogeneity<sup>3</sup> and subsequent injection with TMV

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RNA unambiguously demonstrates that the synthesis of the 160K protein is stimulated proportionally as a function of the concentration of this tRNA in the oocyte (Figs 2, 3). Injection of tRNA<sup>Tyr</sup> isolated from yeast<sup>4</sup> produces the same results (Figs 2, 3). This finding can also be reproduced *in vitro* by addition of tRNA<sup>Tyr</sup> to a TMV RNA-primed reticulocyte extract<sup>5</sup>. Hence tRNA<sup>Tyr</sup> acts as a natural suppressor tRNA in the production of TMV proteins and is probably responsible for the synthesis of the 160 K protein in tobacco plants as well as in *in vitro* and *in vivo* TMV RNA translation experiments where this protein has always been observed<sup>1,6</sup>. This interpretation is supported by the fact that the readthrough protein is synthesized in different protein synthesizing systems and with different tRNA components, such as the *Xenopus* oocyte<sup>2,6</sup>, wheat germ and reticulocyte extracts<sup>7</sup>, and TMV-infected tobacco plants<sup>8,9</sup>. A minor tRNA<sup>Tyr</sup> eluting at higher NaCl concentrations from the RPC-5 column (Fig. 1) can also induce the synthesis of the readthrough protein<sup>5</sup>, but none of the following purified tRNAs could produce this effect: tRNA<sup>Asp</sup>, tRNA<sup>Asn</sup>, tRNA<sup>Glu</sup>, tRNA<sup>His</sup>, tRNA<sup>Phe</sup> and tRNA<sup>Val</sup>, all from *Drosophila*, and tRNA<sup>Ser</sup> from yeast<sup>5</sup>. Furthermore, the *Xenopus* oocyte is not sensitive to artefacts<sup>2</sup>, such as enhanced synthesis of the 160K protein by addition of spermidine or increasing the Mg<sup>2+</sup> concentration<sup>10</sup>.

Amber and ochre suppressor tRNA<sup>Tyr</sup> preparations contain about 1/8th suppressor tRNA<sup>Tyr</sup> and 7/8ths wild-type tRNA<sup>Tyr</sup>, reflecting the fact that only one of the eight tRNA<sup>Tyr</sup> genes present in yeast has mutated to a suppressor, and that these two kinds of tRNA cannot be separated from each other<sup>11</sup>. Hence, the readthrough induced by tRNA<sup>Tyr</sup>-derived amber or ochre suppressors is due to the superimposed effect of the suppressor tRNA<sup>Tyr</sup> and the wild-type tRNA<sup>Tyr</sup> (Fig. 3). Unambiguous and independent proof that the TMV RNA stop codon can be read by amber and ochre suppressors is provided by the finding that amber and ochre suppressor tRNA<sup>Ser</sup> induce readthrough, whereas wild-type tRNA<sup>Ser</sup> does not<sup>2,10</sup>.

TMV RNA has only been partially sequenced<sup>12</sup>. Unfortunately, the sequence in the region of the 110K protein stop codon is not known. However, there are strong indications for the presence of an amber (UAG) stop codon. Amber and ochre suppressor tRNA<sup>Tyr</sup> and tRNA<sup>Ser</sup> can induce readthrough *in vitro*<sup>2,10</sup> and in the *Xenopus* oocyte<sup>2</sup>; amber tRNA shows a slightly enhanced readthrough activity compared with ochre tRNA<sup>1</sup> (see also Fig. 3); yeast tRNA<sup>Tyr</sup> can misread amber codons in a synthetic mRNA and in a Q<sub>β</sub> coat protein amber nonsense mutant, but not in a Q<sub>β</sub> ochre coat protein nonsense mutant<sup>13</sup>. Thus, the first two letters of the codon must be UA, and UGA is excluded.

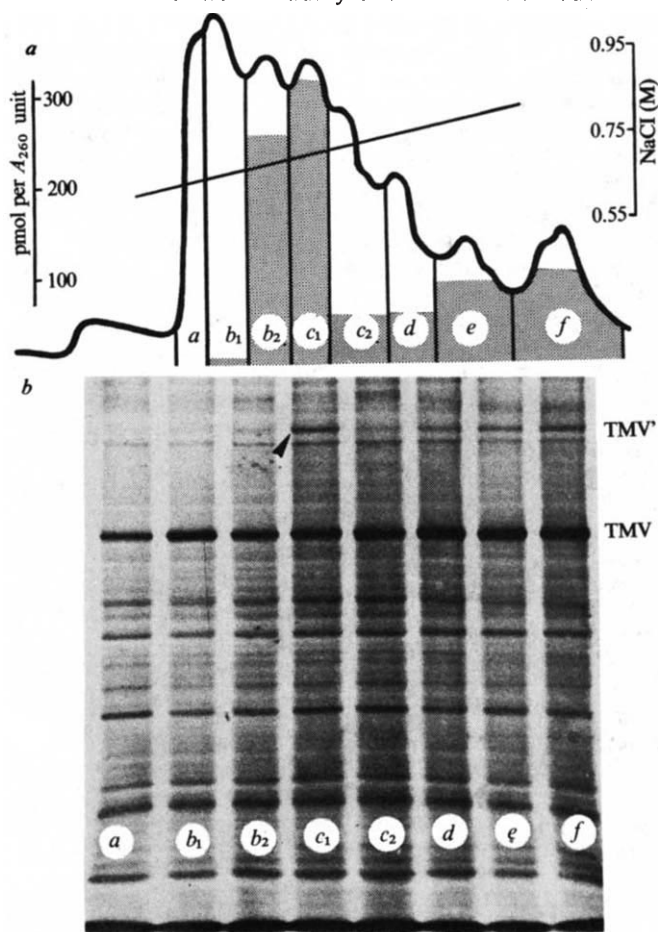
Two other interpretations might be considered: pausing<sup>14</sup>, involving a tyrosine codon as a pseudo stop codon, and out of phase reading<sup>15</sup>. However, pausing seems improbable because termination occurs 10–20 times more frequently at this site than does elongation. Furthermore, it is not obvious why yeast amber and ochre suppressor tRNA<sup>Ser</sup> should enhance the readthrough effect<sup>2,10</sup>, as unconventional base pairing would be required in this case. Out of phase reading also seems improbable because it would lead only to a tyrosine codon (UAU) if the frame is shifted by -2 and if a XUA (Leu, Ile, Ser) codon preceded the stop codon (UXX). It also seems unlikely that the out of phase reading product and the readthrough induced by suppressors would have the same length, which is indeed what is observed<sup>2</sup>.

If an amber codon exists, translation by a wild-type tRNA<sup>Tyr</sup> necessitates a G–G pairing at the third position of the codon, a pairing which is forbidden by the wobble rules<sup>16</sup>. Thus, our results suggest that only the first two bases are relevant for reading through the TMV RNA stop codon, similar to the 'two out of three' reading proposed by Lagerkvist<sup>17</sup>. However, Lagerkvist's hypothesis excludes codons of the UA family from this kind of ambiguous reading.

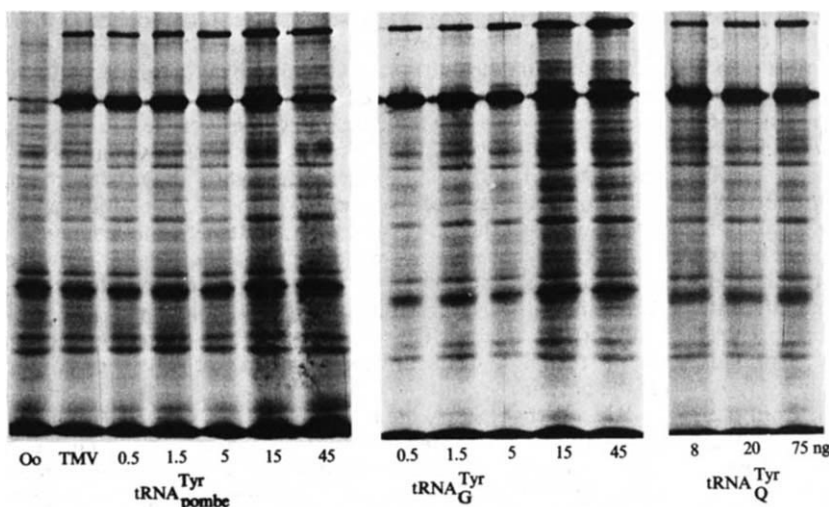
*Drosophila melanogaster* contains two major tRNA<sup>Tyr</sup> isoacceptors<sup>18</sup> (Fig. 1a) which are probably homogeneous and only differ in the presence or absence of the highly modified base Q in

the first position of the anticodon<sup>19</sup>. The full sequence of these isoacceptors has not yet been determined, although preliminary sequencing data confirm this assumption (W. Brunner, M. Altwegg and E. K., unpublished). The tRNA<sup>Tyr</sup>-containing RPC-5 fraction (b<sub>2</sub>) does not stimulate the synthesis of the 160K protein (Figs 1–3). Only very high concentrations of tRNA<sup>Tyr</sup> are able to increase the level of the 160K protein slightly above background (Fig. 3). The presence or absence of the Q base modification does not affect the rate or extent of protein synthesis *in vitro*<sup>20</sup>. The tRNA<sup>Tyr</sup> used in this experiment has not been inactivated (for example, by a nick in the anticodon) because only a single band in the tRNA region can be observed on a 7 M urea polyacrylamide gel<sup>3</sup>. Thus, degree of tRNA<sup>Tyr</sup> modification in the first position of the anticodon regulates the synthesis of the 160K protein.

The fact that eukaryotic tyrosine tRNA is the only one out of 170 sequenced tRNAs that possesses a modified nucleotide in the second letter of the anticodon<sup>21</sup>, a favourable codon context<sup>22–26</sup> and the secondary structure of the TMV RNA



**Fig. 1** Correlation of tyrosine acceptor and readthrough activities of RPC-5 fractions. **A**, Absorption profile ( $A_{260}$ ) of a RPC-5 chromatography<sup>31</sup> (gradient 0.55–0.90 M NaCl) of 200  $A_{260}$  units of crude wild-type *Drosophila* tRNA. The tRNA<sup>Tyr</sup> content of fractions a–f was determined by aminoacylation with <sup>14</sup>C-tyrosine<sup>19</sup> (shaded area, given in pmol per  $A_{260}$  unit). The two major tRNA<sup>Tyr</sup> isoacceptors are localized in fraction b<sub>2</sub> (tRNA<sup>Tyr</sup>) and c<sub>1</sub> (tRNA<sup>Tyr</sup>), respectively. A minor tRNA<sup>Tyr</sup> isoacceptor is found in fractions e and f. **B**, Autoradiography of a 7.5% SDS-polyacrylamide gel of <sup>35</sup>S-methionine-labelled *Xenopus laevis* oocyte proteins after injection of TMV RNA and RPC-5 fractions a–f. The probes were precipitated with ethanol after RPC-5 chromatography, dissolved in 0.1 M Na acetate and reprecipitated with ethanol to remove the salt. The tRNA concentration injected per oocyte was 0.6–1.2  $\mu$ g, the TMV RNA concentration 100 ng. At least seven oocytes were injected for each tRNA fraction. Labelling with <sup>35</sup>S-methionine, extraction of the proteins and SDS-polyacrylamide gel electrophoresis was done as described earlier<sup>2</sup>. TMV = 110K TMV protein; TMV' = 160K TMV readthrough protein. Readthrough activity above background level is found in fraction c<sub>1</sub> containing tRNA<sup>Tyr</sup> (anticodon GΨA). Fraction b<sub>2</sub> containing the Q base-modified tRNA<sup>Tyr</sup> (anticodon QΨA) isoacceptor does not induce readthrough above background level. A slight increase of readthrough activity is associated with the minor tRNA<sup>Tyr</sup> isoacceptor in fractions e and f.

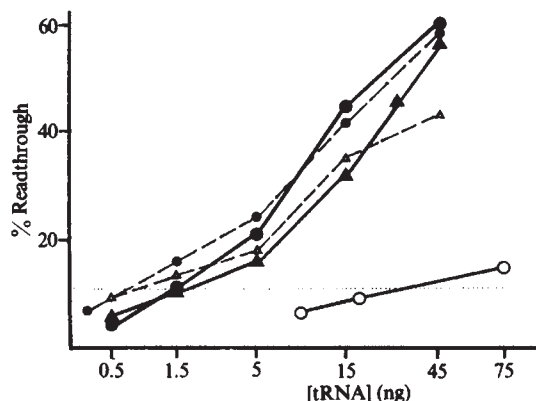


**Fig. 2** Autoradiography of 7.5% SDS-polyacrylamide gels of  $^{35}\text{S}$ -methionine-labelled *Xenopus* oocyte proteins after injection of TMV RNA and various concentrations of  $\text{tRNA}^{\text{Tyr}}$  isolated from *Schizosaccharomyces pombe* and *D. melanogaster* respectively. Experimental procedures are as described in Fig. 1 legend. Synthesis of the 160K TMV readthrough protein is stimulated proportionally as a function of the concentration per oocyte of  $\text{tRNA}^{\text{Tyr}}_{\text{pombe}}$  or  $\text{tRNA}^{\text{Tyr}}_{\text{Drosophila}}$  (anticodons G $\Psi$ A), respectively, whereas  $\text{tRNA}^{\text{Q}}_{\text{Drosophila}}$  (anticodon Q $\Psi$ A) isolated from *Drosophila*<sup>3</sup> is unable to induce this readthrough event. All three tRNAs were at least 95% pure<sup>3,5</sup>.

might contribute to production of appreciable amounts of the 160K protein. These circumstances might also be responsible for the approximately equal efficiency in the reading of the TMV RNA stop codon by wild-type  $\text{tRNA}^{\text{Tyr}}$  and amber or ochre suppressor  $\text{tRNA}^{\text{Tyr}}$  (Fig. 3 and ref. 2). From the above we conclude that the leakiness of the TMV RNA stop codon is due to the misreading of an amber stop codon by a wild-type  $\text{tRNA}^{\text{Tyr}}$ . This is analogous to readthrough of the UGA termination codon of the coat protein of phage QB, thus producing a product indispensable for infection in *Escherichia coli*<sup>27-29</sup>.

Geller and Rich<sup>30</sup> have proposed that the readthrough phenomenon might represent a control of gene expression at the level of termination. They pose the question of whether readthrough of certain proteins occurs, for example, in certain developmental stages of an organism, or after transformation of cells. In fact, significant changes can be observed in the extent of Q base modification in tRNAs isolated from different ontogenetic stages in *D. melanogaster*<sup>19,31</sup>. In transformed mammalian cells the Q base modification disappears almost completely<sup>32</sup>. Whether these changes reflect any activities, as suggested by Geller and Rich<sup>30</sup>, is not known. Putative changes in the extent of Q base modification of the tRNAs in TMV-infected tobacco plants have yet to be demonstrated. Thus, gene expression could be regulated by a single modification enzyme.

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**Fig. 3** Dependence of the amount of the 160K TMV readthrough protein on the concentration of various tRNAs. Experimental procedures are as described in Fig. 1 legend and by M.B. *et al.*<sup>2</sup>. ●,  $\text{tRNA}^{\text{Tyr}}$  (anticodon G $\Psi$ A) from *Drosophila*<sup>3</sup>; ▲,  $\text{tRNA}^{\text{Tyr}}$  (anticodon G $\Psi$ A) from *S. pombe*<sup>4</sup>; ■, *Saccharomyces cerevisiae* amber suppressor  $\text{tRNA}^{\text{Tyr}}$  (ref. 2); △, *S. cerevisiae* ochre suppressor  $\text{tRNA}^{\text{Tyr}}$  (ref. 2); ○,  $\text{tRNA}^{\text{Q}}$ .

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1. Pelham, H. R. B. *Nature* **272**, 469-471 (1978).
2. Bienz, M. *et al. Nucleic Acids Res.* **8**, 5169-5178 (1980).
3. Dudler, R. *et al. Chromosoma* **84**, 49-60 (1981).
4. Vögeli, G. *Nucleic Acids Res.* **7**, 1059-1065 (1979).
5. Bienz, M. thesis, Univ. Zürich (1981).
6. Knowland, J. *Genetics* **78**, 383-394 (1974).
7. Pelham, H. R. B. & Jackson, R. J. *Eur. J. Biochem.* **67**, 247-256 (1976).
8. Sakai, F. & Takebe, I. *Molec. gen. Genet.* **118**, 93-96 (1972).
9. Bruening, G. *et al. Virology* **71**, 498-517 (1976).
10. Morch, M. D. & Benicourt, C. *Eur. J. Biochem.* **105**, 445-451 (1980).
11. Piper, P. W. *et al. Nature* **262**, 757-761 (1976).
12. Zimmern, D. *Cell* **11**, 463-482 (1977).
13. Heckman, J. thesis, Massachusetts Inst. Technol. (1975).
14. Manley, J. L. *J. molec. Biol.* **125**, 407-432 (1978).
15. Atkins, J. F. *Cell* **18**, 1119-1131 (1979).
16. Crick, F. H. C. *J. molec. Biol.* **19**, 548-555 (1966).
17. Lagerkvist, U. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1759-1762 (1978).
18. Twardzik, D. R. *et al. J. molec. Biol.* **57**, 231-245 (1971).
19. White, B. N. *et al. J. molec. Biol.* **74**, 635-651 (1973).
20. Owenby, R. K. *et al. Mechanisms Ageing Dev.* **11**, 91-103 (1979).
21. Gauss, D. H. & Sprinzl, M. *Nucleic Acids Res.* **9**, r1-r23 (1981).
22. Salser, W. *et al. Cold Spring Harb. Symp. quant. Biol.* **34**, 513-520 (1969).
23. Feinstein, S. I. & Altman, S. *Genetics* **88**, 201-219 (1977).
24. Bossi, L. & Roth, J. R. *Nature* **286**, 123-127 (1980).
25. Bienz, M. *et al. Nucleic Acids Res.* **9**, 3835-3850 (1981).
26. Kohli, J. & Grosjean, H. *Molec. gen. Genet.* **182**, 430-439 (1981).
27. Weiner, A. M. & Weber, K. *Nature new Biol.* **234**, 206-209 (1971).
28. Weiner, A. M. & Weber, K. *J. molec. Biol.* **80**, 837-855 (1973).
29. Hofstetter, H. *et al. Biochim. biophys. Acta* **374**, 238-251 (1974).
30. Geller, A. I. & Rich, A. *Nature* **283**, 41-46 (1980).
31. Hosbach, H. A. & Kubli, E. *Mechanisms Ageing Dev.* **10**, 141-149 (1979).
32. Nishimura, S. in *Transfer RNA: Structure, Properties and Recognition*, 59-79 (Cold Spring Harbor Laboratory, New York, 1979).

## ***E. coli* ribosomal protein L10 inhibits translation of L10 and L7/L12 mRNAs by acting at a single site**

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The genes coding for ribosomal proteins L10 and L7/L12 and for the subunits for RNA polymerase  $\beta$  and  $\beta'$  are clustered within the same transcription unit ( $\beta$  operon) located at the 88-min region of the *Escherichia coli* genome<sup>1,2</sup>. The regulation of these genes is of particular interest because of the ratio of synthesis of the proteins (copy ratio L10:L7/L12: $\beta$ : $\beta'$  = 1:~4:0.2:0.2) and because synthesis of the ribosomal proteins and of  $\beta$  and  $\beta'$  is apparently subject to different regulatory signals (see ref. 3 for a review). It was previously shown that certain ribosomal proteins, including L10, can inhibit the translation of their own mRNA and of mRNA of other genes from the same transcription unit<sup>4-11</sup>. This regulation is physiologically significant in coordinating the synthesis of ribosomal proteins and ribosomes. Although L10 was previously shown to inhibit its own translation *in vitro*<sup>5</sup>, the mechanisms for regulation of L7/L12 synthesis have remained unknown. We report here the results of *in vivo* and *in vitro* investigations demonstrating that L10 regulates L7/L12 synthesis, as well as its own synthesis, by acting near the L10 translational start site.



We examined the feedback regulatory properties of L10 *in vitro* using a DNA-directed protein synthesizing system with various DNA templates containing all or portions of the  $\beta$  operon (Fig. 1). When a template containing the entire  $\beta$  operon ( $\lambda$ rif<sup>d</sup>18) was used, addition of purified L10 to *in vitro* reaction mixtures specifically inhibited synthesis of L10 and L7/L12 but did not inhibit synthesis of  $\beta$  and  $\beta'$  or of any other protein (Fig. 2, lanes 1–4; Table 1). By transcribing  $\lambda$ rif<sup>d</sup>18 DNA, extracting the mRNA and then using the mRNA to direct protein synthesis, we found, as have others<sup>5</sup>, that the inhibition by L10 occurs at the level of mRNA translation (data not shown). When a *Hind*III DNA fragment containing only the distal portion of the L10 gene, the entire L7/L12 gene and a proximal portion of the  $\beta$  gene was used to direct protein synthesis, addition of L10 did not affect the synthesis of L7/L12 (Fig. 2, lanes 5, 6). We therefore conclude that L10 acts at a single site and inhibits both L10 and L7/L12 synthesis and that this site is in the promoter-proximal portion of the  $\beta$ -operon transcript.

When a template (pNO1525) containing the entire L10 structural gene and only a portion of the L10 leader sequence was used to direct L10 synthesis, addition of L10 caused marked inhibition of L10 synthesis (Table 1). This result indicates that the 5'-proximal 186 nucleotides of the mRNA are not essential for the repressor action of L10. Using two additional DNA templates, it was also demonstrated that the repressor action of L10 does not require the presence of the distal portion of the L10 cistron. The template  $\lambda\beta$ -*trp-lac*1, made up of DNA of a hybrid '*trp-lac*'  $\lambda$  phage carrying the  $\beta$ -operon promoter and the proximal portion of the L10 gene fused in phase to the distal portion of the *trpB* gene, directed synthesis of an L10-*trpB* fusion protein; the synthesis was specifically inhibited by L10 (Table 1). Similarly, specific inhibitory action of L10 was demonstrated using an *in vitro* system in which the synthesis of an L10- $\beta$ -lactamase fusion protein was directed by a plasmid DNA template, pFM20, that includes the promoter and only the proximal portion of the L10 gene fused in phase to the  $\beta$ -lactamase gene. These results, summarized in Table 1, imply that the target site for L10 repressor action is located between a portion of the L10 leader sequences and the proximal portion of the L10 gene, as shown in Fig. 1. This region includes a possible stem-loop structure in the leader mRNA<sup>12</sup> containing four point mutations<sup>13</sup> that prevent L10 synthesis and which were thought to be involved in the feedback regulatory mechanism for regulation of L10 synthesis. However, two such mutants are in a portion of the leader sequence (at positions 1,515 and 1,516) not required for feedback inhibition. Using a similar *in vitro* system, Brot *et al.*<sup>5</sup> found that L10 inhibited translation of L10

**Table 1** Relative synthesis rates of proteins in the presence of L10

| Template                           | Protein synthesized |        |                 |
|------------------------------------|---------------------|--------|-----------------|
|                                    | L10*                | L7/L12 | Control protein |
| $\lambda$ rif <sup>d</sup> 18      | 0.25                | 0.38   | 0.92            |
| <i>Hind</i> III(1,700 bp)          | —                   | 1.05   | —               |
| pNO1525                            | 0.14                | —      | 1.08            |
| $\lambda\beta$ - <i>trp-lac</i> 1† | 0.29*               | —      | 0.94            |
| pFM20                              | 0.53*               | —      | 0.99            |

Proteins were synthesized as described in Fig. 2 legend in the presence and absence of 1.9  $\mu$ M L10. Synthesis of L10 and L7/L12 from  $\lambda$ rif<sup>d</sup>18 and synthesis of L10 from pNO1525 were determined by immunoprecipitation followed by gel electrophoresis. Syntheses of all other proteins were determined directly by gel electrophoresis. Fluorograms made using preflashed film were scanned with a Joyce-Loebl densitometer. Ratios of amounts of synthesis obtained in the presence of L10 to synthesis obtained in its absence are shown. Proteins listed as 'control' proteins for each template are:  $\lambda$ rif<sup>d</sup>18,  $\beta\beta'$ ; pNO1525,  $\beta$ -lactamase;  $\lambda\beta$ -*trp-lac*1,  $\beta$ -galactosidase; pFM20, a 35,000-molecular weight  $\beta$ -lactamase-L10 fusion protein containing the N-terminus of  $\beta$ -lactamase and the C-terminus of L1.

\* For  $\lambda\beta$ -*trp-lac*1 and pFM20, synthesis of the L10-*trpB* and L10- $\beta$ -lactamase fusion proteins was measured. The proteins of molecular weights 57,000 and 13,000 respectively were identified using anti-L10 antisera.

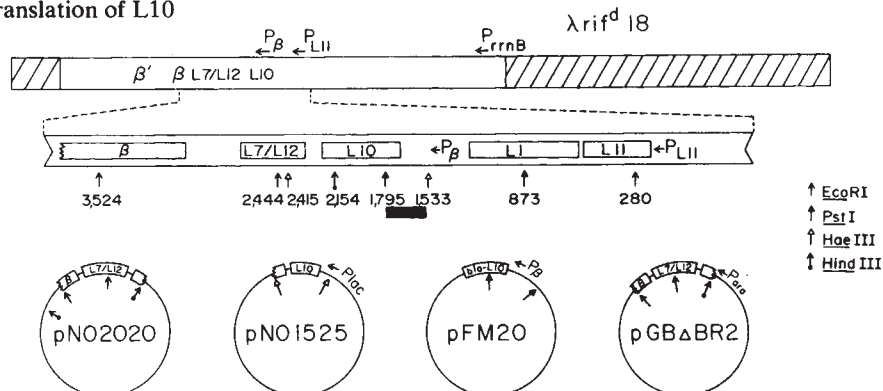
†  $\lambda\beta$ -*trp-lac*1 was constructed by H. de Boer by fusing the *Hind*III-*Eco*RI fragment of  $\lambda$ rif<sup>d</sup>18 containing the  $\beta$  promoter (nucleotides 280–2,154 in Fig. 1) to the left arm of *Hind*III-digested '*trp-lac*' phage  $\lambda$ RS205-7 and to the right arm of *Eco*RI-digested  $\lambda$  phage 459. This method has been described for other ribosomal promoters<sup>27</sup>. The *Hind*III cleavage site in the *trpB* gene<sup>28</sup> is in the same reading frame as the *Hind*III site in the L10 gene.

mRNA but had "little or no effect" on L7/L12 synthesis. We obtained maximal inhibition of L7/L12 synthesis by incubating the reaction mixtures at 30 °C. At 37 °C, our results were more similar to those of Brot *et al.*, showing only 20–30% maximal specific inhibition of L7/L12 synthesis by L10 (data not shown). We believe that our results obtained *in vitro* at 30 °C reflect regulation *in vivo* because of the following results *in vivo*.

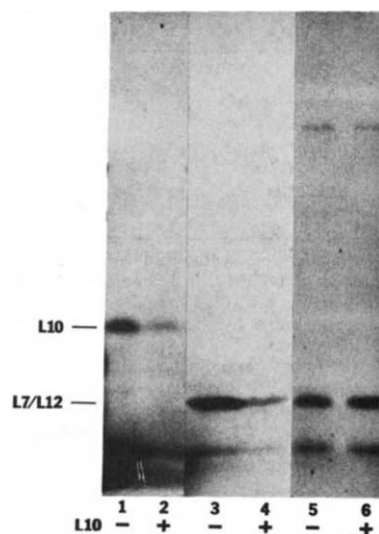
A complex containing four molecules of L7/L12 and one molecule of L10, which is formed on or off the ribosome<sup>14,15</sup>, will inhibit the *in vitro* synthesis of both L10 and L7/L12 by ~50%<sup>16</sup>. We have isolated the L10-L7/L12 complex and found its *in vitro* regulatory properties identical to those of L10 alone (data not shown). Thus, the inhibition of synthesis of L10 and L7/L12 by the complex must be due to the L10 within it. The same conclusion was reached by M. Johnson, T. Christensen and N. P. Fiil (personal communication). Interaction of L10 with the target on the mRNA seems to involve the part of L10 which interacts with 23S rRNA, and not the part that interacts with the four molecules of L7/L12.

To confirm that the regulatory properties observed for L10 *in vitro* reflect its regulatory role *in vivo*, the effect of over-

**Fig. 1** Genes carried by  $\lambda$ rif<sup>d</sup>18 and construction of plasmids used in this study. The hatched regions of  $\lambda$ rif<sup>d</sup>18 represent  $\lambda$  DNA and the open regions bacterial DNA. Some of the bacterial transcription units are indicated above  $\lambda$ rif<sup>d</sup>18 as a P (promoter) and a horizontal arrow indicating the direction of transcription. Transcription of the  $\beta$  operon initiates approximately at position 1,348 ( $\pm$ 1) (ref. 12). Below  $\lambda$ rif<sup>d</sup>18 is an expanded portion of the transducing phage that denotes the relevant bacterial region. Various restriction enzyme sites are indicated below the expanded region. Only the restriction enzyme sites relevant to the present study are indicated. The complete DNA sequence of this region has been determined and can be found in refs 12, 22. The numbers below the vertical arrows representing restriction enzyme sites indicate the nucleotide position of the restriction enzyme cut using the numbering system of ref. 12. Various portions of  $\lambda$ rif<sup>d</sup>18 were used to construct hybrid plasmids (shown in the figure) that carry regions of the L11 and  $\beta$  operons. Some of the restriction sites are shown on the plasmids so that the cloned region can be compared with  $\lambda$ rif<sup>d</sup>18. Plasmid pNO2020 is a derivative of pVH51 (ref. 23) and contains two copies of an *Eco*RI fragment (nucleotides 280–2,444) from  $\lambda$ rif<sup>d</sup>18 and one copy of another *Eco*RI fragment from  $\lambda$ rif<sup>d</sup>18 (nucleotides 2,444–3,524). For *in vitro* studies, the *Hind*III fragment that contains the L7/L12 gene was purified from pNO2020 and used to direct protein synthesis. Plasmid pRZ4006 was used as the parental plasmid for construction of pNO1525. The plasmid pRZ4006 was constructed by K. Bertrand and is a derivative of pBR322 which has the *Bam*HI-*Eco*RI fragment from pBR322 replaced with a 204-base pair (bp) *Hae*III fragment that contains the *lac* operator and promoter (personal communication). Both the *Bam*HI and *Eco*RI sites were retained in pRZ4006 and transcription originating from the *lac* promoter is directed towards the *Bam*HI site. The plasmid pNO1525 was constructed by digesting pRZ4006 with *Bam*HI, treatment with DNA polymerase I and ligation with the *Hae*III fragment containing the L10 gene purified from  $\lambda$ rif<sup>d</sup>18. The resulting plasmid places the synthesis of plasmid-encoded L10 under the control of the *lac* regulatory elements. Plasmid pFM20 was constructed by ligating *Pst*I-digested pBR322 to a *Pst*I fragment (nucleotides 873–1,795) derived from  $\lambda$ rif<sup>d</sup>18. Plasmid pFM20 contains the  $\beta$  promoter and a portion of the L10 gene fused 'in phase' to the  $\beta$ -lactamase gene (*bla*) contained on pBR322 (ref. 24). Plasmid pGB $\Delta$ BR2 was constructed by G. Barry and is described in ref. 17. The site for repressor action by L10 is indicated by a thick bar below the restriction enzyme site arrows.



**Fig. 2** Inhibition of *in vitro* synthesis of L10 and L7/L12 by addition of purified L10. L10 was purified by phosphocellulose chromatography<sup>25</sup>, starting with a 1 M NH<sub>4</sub>Cl/50% EtOH wash of 70S ribosomes depleted of L7/L12 (ref. 26). L10 was stored in 6 M urea, 50 mM methylamine phosphate pH 6.5 at -70 °C. For use in *in vitro* experiments an aliquot of L10 (0.72 µg ml<sup>-1</sup>) was supplemented with bovine serum albumin (BSA; 1.0 mg ml<sup>-1</sup>) and dialysed first against 0.5 M KCl/4 M urea/2 mM potassium phosphate pH 6.5/1 mM dithiothreitol and then against KUP buffer (1.0 M KCl/1 M urea/2 mM potassium phosphate pH 6.5/1 mM dithiothreitol). *In vitro* reaction mixtures (40 µl) received either 2 µl of the resulting L10 solution (lanes 2, 4, 6) or 2 µl of KUP buffer containing 1 mg ml<sup>-1</sup> BSA (lanes 1, 3, 5). Proteins were synthesized in the presence of <sup>35</sup>S-methionine using 0.0017 µM *λ*rif<sup>r</sup>18 DNA (lanes 1-4) or 0.009 µM *Hind*III 1,700-bp fragment (lanes 5, 6) as template, as described previously<sup>19</sup> except that incubation was for 2 h at 30 °C. The *Hind*III 1,700-bp fragment was prepared from pNO2020 (see Fig. 1). After incubations were completed, L10 was added to samples 1, 3 and 5. Reaction mixtures were analysed either directly by gel electrophoresis on a 13% polyacrylamide-SDS gel (lanes 5, 6) or by immunoprecipitation using rabbit anti-L10 antisera (lanes 1, 2) or anti-L7/L12 antisera (lanes 3, 4) followed by gel electrophoresis and fluorography<sup>19</sup>. A fluorogram is shown.



production of L10 *in vivo* was examined. The *Hae*III restriction enzyme fragment that contains the entire L10 gene and a small portion of the L7/L12 gene was inserted into a plasmid vector containing the *lac* operator and promoter so that synthesis of L10 was controlled by *lac* regulatory elements (pNO1525; see Fig. 1). The synthesis of L10 was stimulated by the addition of isopropyl thio-β-galactoside, an inducer of the *lac* operon, in a strain which harbours the recombinant plasmid, and the synthesis rates of other individual proteins were then analysed.

The overproduction of plasmid-encoded L10 caused a marked inhibition of L7/L12 synthesis, and presumably chromosome-directed L10 synthesis, but had no effect on β and

β' synthesis (Table 2). The inhibition of L7/L12 synthesis was due only to L10 overproduction because no such inhibition was observed when the strain carried a deletion within the plasmid-encoded L10 gene (data not shown). To test for a possible *in vivo* regulatory role of L7/L12, we used plasmid pGBΔBR2 which has L7/L12 gene expression under control of *ara* regulatory elements<sup>17</sup>. Addition of L-arabinose to a strain harbouring the hybrid plasmid caused an eight- to ninefold increase in the rate of L7/L12 synthesis but did not affect that of L10 or β and β' (Table 2). (We and others<sup>5</sup>, observed that in the *in vitro* system, purified L7/L12 does not inhibit synthesis of L10 or of L7/L12. We therefore conclude that L10 and L7/L12 are in a translational unit regulated by L10.)

How might L7/L12 synthesis be repressed by L10 acting at the L10 translational start site? One possible explanation is that efficient translation of L7/L12 mRNA requires the translation of the preceding L10 mRNA and that the inhibition of L7/L12 synthesis by L10 is a consequence of the inhibition of L10 synthesis by L10. Such 'translational coupling' has been observed in both the *trp* operon<sup>18</sup> and the L11 ribosomal protein operon<sup>19</sup>. Ribosomal proteins seem to be synthesized from other operons in units of translational regulation with the feedback repressors acting at single mRNA target sites. We have previously suggested a mechanism of sequential mRNA translation to account for the co-regulation and equimolar synthesis of ribosomal proteins in these regulatory units<sup>19</sup>. L7/L12 differs from other ribosomal proteins in that its synthesis is at least four times faster. We suggest that translation of L10 mRNA by a ribosome 'opens' the structure of L7/L12 mRNA initiation site, allowing other ribosomes to initiate the translation of L7/L12 at least four times as efficiently as that of L10 mRNA. We might expect that, without independent feedback regulation of L7/L12 synthesis by itself, more than the four copies of L7/L12 needed per ribosome might always be synthesized. In fact there are reports that, unlike other ribosomal proteins, significant amounts of L7/L12 exist in cells as free proteins<sup>20,21</sup>. Regardless of the exact stoichiometry of the rates of L7/L12 synthesis relative to L10, the present work suggests a mechanism for the co-regulation of the synthesis of L7/L12 and L10.

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- Linn, T. G. & Scaife, J. *Nature* **276**, 33-37 (1978).
- Yamamoto, M. & Nomura, M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3891-3895 (1978).
- Post, L. E. & Nomura, M. in *Ribosomes: Structure, Function and Genetics* (eds Chambliss, G. et al.) 641-669 (University Park Press, Baltimore, Maryland, 1980).
- Yates, J. L., Arfsten, A. E. & Nomura, M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1837-1841 (1980).
- Brot, N., Caldwell, P. & Weissbach, H. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2592-2595 (1980).
- Dean, D. & Nomura, M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3590-3594 (1980).
- Dean, D., Yates, J. L. & Nomura, M. *Nature* **289**, 89-91 (1981).
- Yates, J. L. & Nomura, M. *Cell* **21**, 517-522 (1980).
- Zengel, J. M., Mueckl, D. & Lindahl, L. *Cell* **21**, 523-535 (1980).
- Dean, D., Yates, J. L. & Nomura, M. *Cell* **24**, 413-420 (1981).
- Wirth, R., Böck, A. *Molec. gen. Genet.* **178**, 479-481 (1980).
- Post, L. E. et al. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1697-1701 (1979).
- Fill, N. P., Friesen, J. D., Downing, W. L. & Dennis, P. P. *Cell* **19**, 837-844 (1980).
- Subramanian, R. A. *J. molec. Biol.* **95**, 1-8 (1975).
- Marquis, D. & Fahnestock, S. J. *molec. Biol.* **119**, 557-567 (1978).
- Fukuda, R. *Molec. gen. Genet.* **178**, 483-486 (1980).
- Barry, G., Squires, C. L. & Squires, C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4922-4926 (1979).
- Oppenheim, D. S. & Yanofsky, C. *Genetics* **95**, 785-795 (1980).
- Yates, J. L. & Nomura, M. *Cell* **24**, 243-249 (1981).
- Morrissey, J. J., Weissbach, H. & Brot, N. *Biochem. biophys. Res. Commun.* **65**, 293-301 (1975).
- Morrissey, J. J., Cupp, L. E., Weissbach, H. & Brot, N. *J. biol. Chem.* **251**, 5516-5521 (1976).
- Delcuve, G., Downing, W., Lewis, H. & Dennis, P. P. *Gene* **11**, 367-373 (1980).
- Herschenfeld, V., Boyer, H. W., Chow, L. & Helinski, D. R. *J. Bact.* **126**, 447-453 (1976).
- Stutcliffe, J. G. *Cold Spring Harb. Symp. quant. Biol.* **43**, 77-90 (1979).
- Zimmermann, R. A. & Stöffler, G. *Biochemistry* **15**, 2007-2017 (1976).
- Hamel, E., Koka, M. & Nakamoto, T. *J. biol. Chem.* **247**, 805-814 (1972).
- Miura, A., Krueger, J. H., Itoh, S., de Boer, H. A. & Nomura, M. *Cell* **25**, 773-782 (1981).
- Crawford, I. P., Nichols, B. P. & Yanofsky, C. *J. molec. Biol.* **142**, 489-502 (1980).
- Clark, D. J. & Maaloe, O. *J. molec. Biol.* **23**, 99-112 (1967).
- Howard, G. A. & Traut, R. R. *FEBS Lett.* **29**, 177-180 (1973).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).

**Table 2** Relative synthesis rates of proteins after induction by isopropyl thio-β-galactoside or L-arabinose

| Protein | Strains                     |                             |
|---------|-----------------------------|-----------------------------|
|         | N02396<br>(carries pNO1525) | N02455<br>(carries pGBΔBR2) |
| S3      | 1.05                        | 0.91                        |
| S4      | 1.00                        | 1.05                        |
| S10     | 0.81                        | 1.15                        |
| L1      | 1.05                        | 1.05                        |
| L2      | 0.96                        | 0.90                        |
| L4      | 1.00                        | 1.17                        |
| L7/L12  | 0.26*                       | 8.9*                        |
| L10     | 1.80*                       | 0.97                        |
| β + β'  | 0.85                        | 0.98                        |

For labelling experiments, cells were grown in a synthetic minimal medium<sup>29</sup> supplemented with 0.4% glycerol, all amino acids (except methionine and lysine) at 50 µg ml<sup>-1</sup> and thiamine at 2 µg ml<sup>-1</sup>. Cells were grown at 37 °C to 2 × 10<sup>8</sup> per ml. Samples of cells were pulse-labelled with <sup>3</sup>H-lysine (646 pmol ml<sup>-1</sup>; 80.5 Ci mmol<sup>-1</sup>) for 1 min followed by a 1-min 'chase' with excess nonradioactive lysine. Cells were labelled before (control cells) and 10 min after addition of isopropyl thio-β-galactoside to 1 mM or 0.2% L-arabinose, depending on the strain used (experimental cells). Cells were rapidly chilled and then mixed with a suitable amount of <sup>14</sup>C-lysine-labelled carrier cells. <sup>14</sup>C-labelled cells were prepared by growing cells in the presence of <sup>14</sup>C-lysine for several generations. Ribosomal proteins were extracted and separated by two-dimensional gel electrophoresis<sup>30</sup>. The β and β' subunits were resolved from other proteins by SDS slab gel electrophoresis<sup>31</sup>. After staining the gels, the spots corresponding to the indicated ribosomal proteins were extracted, the gels oxidized with a Packard sample oxidizer and <sup>3</sup>H and <sup>14</sup>C measured separately. The <sup>3</sup>H/<sup>14</sup>C ratio in each protein in the experimental cells was then compared with that in control cells using the expression:  $\frac{[^3\text{H}/^{14}\text{C}] \text{ in protein } i \text{ in experimental cells}}{[^3\text{H}/^{14}\text{C}] \text{ in protein } i \text{ in control cells}}$

The ratios obtained were then normalized to the ratio obtained for total cellular protein. Values given are the average of two independent experiments.

\* Values significantly different from 1.0.



## BOOK REVIEWS

# Eureka!

## No mystique to creative thinking

Stuart Sutherland

EVERYBODY, from Arthur Koestler to Edward de Bono, knows that there is something very mysterious about creative thinking. To achieve it one must allow one's unconscious mind to work in unusual ways in order to produce that sudden insight that prompted Archimedes to leap from his bath and startle the citizens of Syracuse by running naked through the streets. Unfortunately, the fact that these thought processes cannot be specified has not stopped Arthur Koestler and others of his ilk from writing about them at length and giving them a variety of curious names such as biassociative, Janusian, paleological, homeospatial, divergent and lateral thought. In *The Mind's Best Work*, David Perkins debunks the mystery of creation. Just as a great athlete uses the same muscles as the puniest individual, so the great creators use the same mundane thought processes that mediate everyday existence. To prove his point he draws not merely on the intellectual biographies of such men as Darwin and Coleridge, but on a variety of ingenious experiments undertaken by himself and others, backed up by a series of demonstrations that the reader can try out on himself.

Two of the thought processes common in creative thinking are recognition and noticing. In seeking for a difficult proof, the mathematician may recognize a similarity between the structure of the problem and that of one for which there is an existing proof, thus suggesting a method of approaching the problem. Noticing occurs when some relevant feature of a problem is detected without any conscious attempt to discover it. Just as a woman interested in fashion may notice a flaw in a new costume, so the poet may notice that a word or phrase he has written is weak. What is noticed depends very much on one's interests and knowledge. The mind has a remarkable power to recover from memory items that satisfy very diverse criteria. Anyone can recover from memory the name of a food that is white and soft and whose name begins with an 's', even though he has never been confronted with this combination of criteria before. In the same way someone working on a new problem with particular criteria for solution may discover an answer that will be regarded as original.

Perkins's own research, in which he obtained protocols of subjects' thought

*The Mind's Best Work*. By D.N. Perkins. Pp.368. ISBN 0-674-57627-6. (Harvard University Press: 1981.) \$18.50, £12.95.

processes whilst solving problems, suggests that the mind rarely takes great leaps. It proceeds in a step-by-step fashion through successive acts of recognition and noticing which lead towards a solution. Where a sudden leap is taken, it is because through practice on a particular type of problem someone has learned to recognize a larger pattern that is of significance.

Moreover, creative thought is not wayward — there is a reason for each step taken. The reason can usually be articulated, but not always. In everyday life we may recognize that a friend is looking morose without being able to specify what aspect of his demeanour the judgement is based upon. All creative activity involves noticing flaws, and Perkins found that good painters and poets were better than poor ones at articulating what was wrong with an unfinished work. A gut feeling that something is wrong is less help in putting it right than an ability to specify the nature of the flaw and thus to establish criteria for how to go about improving it.

The role of incubation in the unconscious mind has, according to Perkins, been overplayed. Where it helps — and there is evidence to suggest that it rarely does — it probably does so for one of two reasons. First, one of the main obstacles to solving a problem is the difficulty in giving up a particular approach which is not paying off. A respite from thinking about a problem may make it easier to try a new approach. Second, whilst someone is thinking of something else, he may be lucky enough to encounter a useful hint that could not readily be obtained in the course of a frontal attack. In just this way, Malthus's essay on population gave Darwin the clue he needed to suggest the survival of the fittest as the selective mechanism in evolution. Laboratory experiments clearly establish the importance of such hints, many of which are used by subjects without their being aware that any hint has been provided.

Creativity in a particular field depends, of course, on a thorough knowledge of it and on practice in existing techniques.

Abstraction plays an important role — people must learn that a heuristic that has been found to solve one problem may be used to solve similar problems. Again, the more the heuristics are articulated the more helpful they are. In one experiment, a group of students in a class on integral calculus were shown how the use of five heuristics could help in solving sample problems. A second group were shown how to solve the same initial set of problems, but no effort was made to emphasize the generality of the heuristics used. In testing on further problems, the former group solved twice as many as the latter.

In considering the question of what makes someone creative, Perkins points out that the obvious answer is usually ignored. An important element in creativity is the desire to be creative; one can set oneself to reject the obvious and to search for new solutions to a problem. The standards people set themselves in solving problems can be all important for the solutions they produce. Subjects urged to suggest "a good title for a novel" whose plot they had been given gave solutions that were markedly inferior to those produced by subjects urged to provide "an imaginative, creative or unusual title for this plot". There is also evidence that artists and poets think critically about their own work and a willingness to accept criticism from others correlates with the quality of their achievement. Art students who were open to criticism were found a few years later to be more successful than those who rejected it, though this particular experiment may only show that an artist who wants to please his public will sell more paintings than one who does not care what the public thinks. Because of the importance of self-criticism, geniuses are not necessarily fluent: Beethoven composed with painful deliberation. One of Perkins's more curious findings is that the average rate of composition of pentameters by both good and bad poets is half a line a minute and he claims that even Byron, reputedly one of the most fluent poets, did not exceed this rate in writing *The Corsair*. But if fluency is not a necessity for creation, luck often is. The man must be nicely matched to the problem. Einstein's intolerance for contradictions motivated the special theory of relativity, but the very same trait led him

to waste his later years seeking an alternative to quantum theory.

It has only been possible here to sketch the nature of Dr Perkins's approach to creativity. He develops the points I have made in much more detail and makes many other illuminating suggestions. Although scholarly, his book is intended for the general public and is easy to read; it has only two faults — a tendency to write down to the reader and the inclusion of some singularly unattractive line drawings, which have little relevance to the text and serve to demonstrate only that not all artists are creative. He is of course aware

that reducing the thought processes of creativity to those employed in everyday life does not explain the nature of those thought processes. But this reduction is a good beginning if only because it frees psychologists to get on with the task of explaining ordinary thought processes, and may help to stop other authors from continuing the existing tradition of producing turgid and obscure tomes on the mysteries of creation. □

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## Contradictions in theoretical chemistry

H.C. Longuet-Higgins

*Chemistry, Quantum Mechanics and Reductionism.* By Hans Primas. Pp.451. ISBN 3-540-10696-0. (Springer-Verlag: 1981.) DM 68, \$32.40.

"THE purpose of this book" writes Professor Primas "is to provide a deeper insight into the modern theories of molecular matter. It . . . is not meant to be a textbook; in many respects it has complementary goals. For good and bad reasons, most textbooks ignore the historical and philosophical aspects [of theoretical chemistry] and go ahead on the basis of crude simplifications; many even lie like the Devil and do not shrink from naive indoctrination".

Remarks such as these are well calculated, as the author intends, to ruffle the placid waters of theoretical chemistry. It is difficult enough, in all conscience, to explain the technicalities of quantum chemistry to lively young minds without having one's authority undermined by pronouncements such as "the theoretical basis of chemistry and biology is not safely in our hands". From a scientist of less achievement than Professor Primas such an assertion might well be greeted with open hostility in the community of theoretical chemists. How can it be reconciled with our evident ability to compute, with greater and greater accuracy, the dissociation energies, dipole moments, force constants and other properties of molecules? If the Schrödinger equation is not good enough for Professor Primas, has he a better one to propose?

Such a reaction to the opening pages of *Chemistry, Quantum Mechanics and Reductionism* would entirely miss the author's point. Anyone who has studied and taught theoretical chemistry for any length of time will know how difficult it is

to reconcile, in his own mind and the minds of his students, the elusive properties of sub-atomic particles with the evident permanence of large molecules and crystals. There is an almost irresistible temptation to suppose, and to imply, that there is one set of laws for the very small and another for the large — a proposition which is in flat contradiction with the orthodox interpretation of quantum mechanics. Professor Primas asserts that

The mode of activity exhibited by contemporary science strikingly reminds one of what Shapiro (1965) has called the paranoid style. It is characteristic of this style that bridges between related problems are broken down so that things remain neatly and rigidly separated. Scientists who cultivate a paranoid research style are usually extremely acute and intense, show an exorbitant respect for compartmentalisation and computers, and firmly demand complete autonomy for their narrowly fixed ideas . . . The separation of philosophy and science has led to the so-called restricted world view and to the blindness of many experts who are entirely unaware of the abstract and isolating nature of modern science.

No, these are not the ravings of a iconoclast; in this long and fascinating book (it runs to over 400 pages) the author reveals a deep understanding and appreciation of the most difficult problems of quantum mechanics and of the acute paradoxes associated with its interpretation — or rather, with those alternative interpretations which do not violate experimental fact. He reveals himself as a master not only of the mathematics but also of the philosophy of science, and as a man who cares passionately about intellectual integrity, in the widest possible sense of that word. The book is uncompromisingly difficult, in an area where compromise would be unforgivable; but it is spiced with good plain questions of an acutely embarrassing kind: "What is quantum mechanics about?" "What is a classical description?" "Do isolated quantal systems exist at all?" "Does quantum mechanics apply to large molecular systems?" and so on. I wonder how

most theoretical chemists would answer these questions if some bright student were tiresome enough to ask them!

*Chemistry, Quantum Mechanics and Reductionism* is Professor Primas's own very positive contribution to the solution of some of these problems. The best-known fully-developed interpretation of quantum mechanics is that of von Neumann, London and Bauer, and from this Professor Primas wants to remove just one unproved axiom, namely the universal validity of the superposition principle. He develops an alternative interpretation of his own, based on sophisticated algebraic concepts, of which the most important is that of a "W\*-algebra". To master his ideas would obviously take much time and thought, but they are logically compatible (as the orthodox interpretation is not) with the attribution of "classical" properties to molecules — an assumption which all theoretical chemists use without apology when referring to the charge, the mass or the structure of an ordinary molecule. The book may be difficult, but it is quite unusually honest and thoughtful, and is likely to prove an invaluable antidote to the narrowness of outlook which is so often apparent in chemical theorizing. □

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## Crucial colonization

Barry Cox

*The Terrestrial Environment and the Origin of Land Vertebrates.* The Systematics Association Special Volume, No. 15. Edited by A.L. Panchen. Pp.633. ISBN 0-12-544780-9. (Academic: 1981.) £38, \$91.50.

The earliest known tetrapods are the ichthyostegid amphibians, now known from Late Devonian rocks in both Greenland and Australia. From this beginning sprang a variety of larger "labyrinthodont" amphibians, apparently ancestral to the reptiles, as well as the smaller "lepospondyl" amphibians. The transformation of our interpretation of palaeogeography, as well as progress in such fields as Palaeozoic floras and both invertebrate and vertebrate faunas, makes this a timely volume. The 20 papers were originally given at a symposium in Newcastle upon Tyne in April 1979, which was organized by Alec Panchen, editor of the book.

Two of the papers are concerned with the geography of the world in which the first tetrapods evolved. Tarling analyses palaeomagnetic data and provides a series of continental reconstructions for the Early Devonian to Late Permian; he

The latest supplement to *A Bibliography of Ab Initio Molecular Wave Functions* by W.G. Richards *et al.* has just been published by Oxford University Press. Covering the years 1978–1980, the book costs £35.



suggests that China may have provided a link between Australia and Euramerica. The relationship between the changing Carboniferous geographies and the dispersal of early tetrapods is discussed by Johnson.

Two other papers deal with Palaeozoic botany. Dianne Edwards considers the appearance, habits and affinities of early vascular plants, but concludes sadly that little progress has been made in reconstructing the details of terrestrial plant communities of Devonian times, while Scott reviews the ecology of Late Palaeozoic floras, emphasizing the innovative effects of the evolution of arborescence and seed production. The only other non-vertebrate contribution is that by Rolfe, who describes the early invertebrate terrestrial faunas; these were dominated by arthropods, the larger of which, together with the amphibious eurypterids and scorpions, must have provided a food source for the earliest amphibians.

In one of the most interesting of the vertebrate papers, Thomson points out that there is little evidence that any of the Devonian lobe-finned fishes were primarily freshwater in habitat; he believes that the amphibians may have evolved from fish that still spent at least part of their lives in coastal lowland estuaries. But to which lobe-finned group did this fish belong? Opinions seem to be becoming more, not less, diverse. Though Rackoff's study of the fins of one osteolepid rhipidistian shows a number of close resemblances to the tetrapod limb, Janvier believes that the osteolepid pectoral girdle, though variable, could not have evolved into that of tetrapods. But an even more heretical phylogenetic idea also appeared at the symposium. Both Patterson, in an historical review of the problem of tetrapod origins, and Gardiner in a reappraisal of current evidence, suggest that the lungfish are the closest relatives of the tetrapods. The debate on this central issue promises to be interesting and fruitful.

In another morphological paper, Carroll notes the similarity between the hyomandibular of rhipidistians and that of early amphibians, and suggests that the bone retained a cheek-support function well into the Amphibia, only a few lines having independently transformed it into a sound-transmitting stapes.

Two early tetrapod faunas are reviewed. The new basal Late Carboniferous Scottish Cowdenbeath fauna is described by Smithson; it includes aquatic fish-eating and possibly herbivorous forms, as well as one genus that may have eaten terrestrial invertebrates, but provides few antecedents for later faunas. Andrew Milner makes a detailed analysis of the well-known, very diverse, later Carboniferous fauna of Nýřany, Czechoslovakia, and distinguishes its different components — open-water, shallow swamp-lake and

terrestrial-marginal endemics, as well as rarer elements from neighbouring environments.

Finally, a number of authors discuss the origin and classification of particular taxa — the anthracosaurian amphibians (Panchen), the Nectridea (Angela Milner), the Cotylosauria (Heaton), the Pelycosauria (Reisz) and the major amniote groups (Gaffney).

The contributions maintain a high

standard of interest and readability, and the volume is attractively produced; there are few typographical errors, and there is both an author and a subject index. It should serve for many years as both a benchmark of our knowledge, opinions and attitudes today, and as a source of references to the earlier literature. □

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## Electron microscopy at the limits

David J. Smith

*Experimental High-resolution Electron Microscopy.* By J.C.H. Spence. Pp.420. ISBN 0-19-851365-8. (Clarendon/Oxford University Press: 1981.) £35, \$74.

THE technological revolution of the past decade has resulted in a concomitant improvement in the flexibility and usage of the commercially available electron microscope, not least in the direction of improved performance. Lattice and point resolutions in the region of 1 Å and 3 Å, respectively, have become standard. Hence, instead of high-resolution electron microscopy remaining an esoteric art, it is, in principle at least, now available to all those who can afford the admittedly considerable price of the latest instruments. That more scientists have not yet taken advantage of the opportunity to resolve physical details almost at the atomic level probably reflects the fact that recording a high-resolution micrograph, even with these advanced microscopes, is still difficult; moreover, the literature does not provide much guidance to the newcomer. This readable account of high-resolution methodology thus represents a sorely needed addition to the field.

The book can be considered to consist of two parts. The first, comprised of Chapters 3 and 4, the first half of Chapter 5 and most of Chapter 6, provides a useful introduction to dynamical electron diffraction theory, setting out clearly most of the underlying concepts and in particular emphasizing the possible pitfalls of micrograph interpretation. Copious references have been included, making these chapters useful even to experienced workers. These sections are also carefully cross-referenced to the remainder of the book.

On the experimental side, there are separate chapters on lenses and electron sources; much of the information here is tangential to the major theme of the book, and could well be omitted in a first reading. Nevertheless, these chapters represent valuable compilations of background material and, again, are well-referenced.

The remaining chapters deal with the practical problems involved in realizing

high-resolution micrographs, pointing out the requirements for knowledge of relevant electron-optical parameters, describing possible sources of interference and instability, and discussing operating techniques. These details will be of greatest value to the novice but many experienced workers will undoubtedly also find information of relevance to their research.

Despite the obvious attractions of the book, it is not free of some misleading or incorrect statements, and there are certainly strong arguments for re-ordering the subject matter. For example, the second half of Chapter 5, which briefly describes some applications, could well follow Chapter 10 on methods, and Chapters 1 and 2, which primarily deal with experimental details, would be better placed after the theoretical discussions of Chapter 6. The worst of the factual errors, presumably due to the publisher rather than the author, is located on the dust jacket where it is claimed that "Applications . . . are all described in full practical detail. . ." — this was manifestly not one of the author's objectives. It would also appear unlikely that any manufacturer of lanthanum hexaboride cathodes would readily acquiesce to operation in vacuum levels of only "better than  $10^{-5}$  T" (p.262), and most users of field-emission sources would expect tip lifetimes of the order of several months, irrespective of operating voltage, rather than "about 80 hours" (p.263).

Such criticisms are obviously minor, and overall the author has produced a text which is far more than a "recipe book" for the beginner. He has evidently drawn on the wealth of knowledge of his many friends and colleagues in Oxford, Melbourne and Tempe, three of the leading centres in the field, as well as his own practical experience, and his book should become a valued guide and source of information for the practising microscopist. □

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# Fish-heads and breadcrumbs to feed the five thousand

John Rivers

*Nutrition and Food Science: Present Knowledge and Utilization.* Edited by Walter J. Santos *et al.* Three-volume set. Vol. 1, *Food and Nutrition Programs and Policies*, pp.850, ISBN 0-306-40342-0. Vol. 2, *Nutrition Education, Food Science and Technology*, pp.900, ISBN 0-306-40343-9. Vol. 3, *Nutritional Biochemistry and Pathology*, pp.758, ISBN 0-306-40344-7. (Plenum: 1980.) Vol. 1 \$75, £47.25; Vol. 2 \$79.50, £50.09; Vol. 3 \$69.50, £43.79. Three-volume set \$195, £122.88.

*Nutrition and Food Science* is a weighty work: three volumes, 2,500 pages, 750,000 words. That is about as long as the Bible or the complete works of Shakespeare. It seems reasonable to ask if these three books measure up to such standards.

The answer, sadly, is no. These volumes will rarely be read, and even more rarely enjoyed. They cannot even be treated as a coherent work: they are a patchwork of 250 essays linked by the fact that their authors attended the Eleventh International Congress of Nutrition in Brazil, 1978.

The editors have given each volume a subtitle and subdivided each volume, presumably to impose some order on them, but this attempt fails. For example the title for Vol. 2, *Nutrition Education, Food Science, and Technology*, is in itself a portmanteau. Fittingly so, since the volume is further subdivided into four parts — Animal and Vegetable Resources for Human Feeding, Food Science and Technology, Research in Food Nutrition (*sic*) and Nutrition Education — and since national nutrition surveys are put in Vol. 2 even though Vol. 1 seems more appropriate.

In this electronic age the international congress is becoming an anachronism. In small fields, especially fast-moving ones, it is still a feasible and important way of communicating. But all too often congresses are only "holidays for the boys". The cliché that the best science is discussed in the bar is actually a two-edged one.

Some time ago the *Economist* magazine, in an article on scientific research, noted that, of the 32 participants at an international physics conference in 1927, a third were, or were to be Nobel Laureates. An equivalent meeting in 1980 had 800 participants, no Nobel prizewinners and only routine investigations were reported. The *Economist* should have dissected nutrition. The triennial International Nutrition Congresses have doubled in size about every ten years. The science they represent grows only one-quarter as fast, so necessarily the congresses have become less and less important as a legitimate part of scientific communication, and more and more a social event.

At the Eleventh Congress, 5,000

participants gathered together in Brazil and seem to have achieved nothing: were all those journeys really necessary? Like a somewhat more famous gathering of 5,000 they subsisted on a very meagre diet. But if a miraculous transformation of the loaves and fishes of their intellects occurred, then it must be the baskets of leftover rubbish that have been published for posterity.

For that is what this work is about. It is a three-volume commemoration of an event. There are some good, original papers, such as Jul's study on dairy farming in Calcutta, but they are few, and will probably be lost in the mound of fish-heads and breadcrumbs. A mound of papers in which nothing much is said, in which most tables

and graphs seem to be referred back to previous publications, in which representatives of commercial organizations are simply reporting the use of their products, and a collection which overall could have done with refereeing and extensive subediting.

*Nutrition and Food Science* is a reverberating cry for something to be done to stem the flow of paper that is dignified by the appellation "scientific literature". This is neither science nor literature. □

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## Depth in stability for the cognoscenti

P.H. Roberts

*Hydrodynamic Stability.* By P.G. Drazin and W.H. Reid. Pp.525. ISBN 0-521-22798-4. (Cambridge University Press: 1981.) £35, \$77.

"Not every solution of the equations of motion, even if it is exact, can actually occur in nature. The flows that occur in nature must not only obey the equations of fluid dynamics, but also be stable". The significance of this apt quotation from Landau and Lifshitz with which the authors of *Hydrodynamic Stability* open their book is evident from the scarcity in nature of simple orderly ("laminar") motions and the abundance of irregular ("turbulent") flows. The transition from the former to the latter starts with the instability of the laminar flow, a process that can be studied in controlled laboratory conditions and, with comparative ease, by theoreticians.

The achievements of stability theorists are now so extensive that the authors had to make hard decisions in limiting their text to even 525 pages. They have opted for depth, rather than breadth, of treatment, and paid particular attention to classic situations such as Bénard convection, circular (Couette) flow and plane parallel flow.

A consideration in the authors' choice of topics was probably the existence of Chandrasekhar's classic work, *Hydrodynamic and Hydromagnetic Stability* (Clarendon Press, 1961). Wonderful though Chandrasekhar's treatise is, it is sometimes felt today that it contains a number of lacunae: it overstates variational methods; it ignores a useful technique (asymptotic analysis); it omits one of the oldest and possibly the mathematically most interesting of all linear stability problems, that raised by

plane Poiseuille flow; and it says little about non-linear stability. None of these points would have been fair criticism in 1961 when the book was published, but today many specialists will welcome Drazin and Reid's account, if for no better reason than because it successfully redresses the balance.

The authors are of course well known, particularly for their work on parallel flows, and it is here that readers will discover the most penetrating discussions, and the deepest and most elegant mathematics. Indeed, some may wonder whether the authors' enthusiasm has carried them too far, and whether all of the sophisticated theoretical weapons displayed are essential to their own armouries. This space might have been better used in expounding non-linear theory still further, or in providing deeper discussions of baroclinic instabilities, another subject that has exploded since Chandrasekhar wrote his book. Most readers will find the discussion of pinch instabilities frustratingly brief.

These are minor reproaches only. The work is undeniably of high scholarship, consummate accuracy and penetrating insight. Teachers of graduate courses will find the problems at the end of each chapter imaginative, but difficult: solutions can be obtained by application to either author. All specialists in stability theory will be happy that two such authorities have found the time, and spared so few pains, to produce a work of such excellence. □

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